DIVERSITY IN THE REGULATION OF METABOLIC FUEL USE IN MICE SELECTED FOR HIGH LOCOMOTOR ACTIVITY

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ABSTRACT

Despite diversity in locomotion, the mammalian pattern of metabolic fuel use appears highly conserved, and in most circumstances relative exercise intensity dictates the proportionate contribution of carbohydrates and lipids to energy supply. However, the mechanistic explanations for unity of fuel use patterns are not known. The aim of my study was to determine if fuel use during exercise could be adaptively altered or differentially regulated in animals selected for high activity.

To this end, I used a model of experimental evolution in which mice are selectively bred for high locomotion. Within this model, there are two distinct phenotypes of high running (HR) mice: HR_{mini} mice, characterized by half-sized hindlimb muscles with increased mass-specific aerobic capacity, and HR_{normal} mice, with a normal muscle phenotype. I evaluated aerobic capacity (VO_{2max}), fuel use during exercise, cardiac properties, and physiological factors involved in regulating fuel use, in one line each of: 1) non-selected control mice, 2) HR_{mini} mice, and 3) HR_{normal} mice.

HR mice exhibited an increased VO_{2max} compared to controls; moreover, HR_{mini} mice had a greater VO_{2max} than HR_{normal} mice. Metabolic and physical cardiac changes may have contributed to these VO_{2max} differences. However, HR_{mini}, HR_{normal}, and control mice did not differ in the mixture of fuels supplying energy demand, when exercise intensity was scaled to VO_{2max}. HR mice achieved enhanced absolute fuel oxidation rates via different means. HR_{mini} mice had increased cytosolic fatty acid binding protein (H-FABP), fatty acid transporter (*FAT/CD36*) mRNA, peroxisome proliferator-activated receptor (*PPAR*) α mRNA, and activities of aerobic enzymes in skeletal muscle, as well as a different muscle recruitment pattern. Conversely, HR_{normal} mice had enhanced whole-muscle enzyme activities. Therefore, there are multiple mechanisms to enhance fuel oxidation rates with an elevated VO_{2max}, and diverse mammals can differentially utilize these mechanisms without deviating from a conserved pattern of fuel use.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format, as recommended and approved by members of my supervisory committee. It consists of three chapters. Chapter one is a general introduction and overview of background material and the objectives of work. Chapter two consists of a discrete manuscript that is in preparation for submission to a peer-reviewed scientific journal. Lastly, chapter three summarizes the major findings of this thesis, places these findings in the context of current knowledge, and indicates future directions that this research might take.

Chapter 1: **General introduction**

Chapter 2: **Diversity in the regulation of metabolic fuel use in mice selected for high locomotor activity**

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Chapter 3: **General summary and conclusions**

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CHAPTER 1: GENERAL INTRODUCTION

One of the fundamental requirements of survival for any organism is the balance of ATP production and utilization. Exercise, for instance, requires a great deal of energy, primarily consumed by skeletal muscle (Taylor, 1987). This necessitates an increase in ATP production, which is achieved via metabolism of fuels that have either been ingested or stored within the body, primarily by aerobic metabolic pathways and oxidative phosphorylation (Hochachka and Somero, 2002).

Along with ensuring adequate oxygen delivery, a major component of maintaining energetic homeostasis during periods of increased ATP demand is the coordinated ability to both increase total flux of substrates through metabolic pathways and adjust the proportionate mixture of fuels contributing to total oxidation (Hochachka and Somero, 2002; Weber and Haman, 2004). The appropriate mixture of fuels is influenced by the metabolic properties of the fuels themselves. For instance, lipids are stored in large quantities in the body because they are highly reduced and anhydrous, thus providing the most light-weight, concentrated source of stored energy (Hochachka and Somero, 2002; Weber and Haman, 2004). Carbohydrates, on the other hand, are required for immediate and/or high rates of ATP production, and can support anaerobic metabolic pathways, unlike lipids (Brooks and Mercier, 1994; Hochachka and Somero, 2002; Weber and Haman, 2004). Therefore, lipids seem to be the most appropriate of these fuels for supporting endurance locomotion, whereas carbohydrates have many properties that are important for exercise performance at a high intensity or when oxygen is limited (*i.e.* hypoxia; Weber and Haman, 2004).

Despite apparent advantages of adjusting preferred utilization of these substrates with adaptive or environmental variation, the mammalian pattern of fuel use during exercise appears to be highly conserved, and in most circumstances relative exercise intensity dictates the proportionate contribution of carbohydrates and lipids to energy supply (McClelland, 2004). However, the hypothesis that lipids would be favored in animals adapted for endurance exercise has not been fully tested. In my study, I will use a model of experimental evolution to determine whether the pattern of whole-body metabolic fuel use during exercise can be adaptively altered or differentially regulated when animals are under selection for high locomotor activity.

Fuel use in exercising mammals

In mammals, lipids and carbohydrates are the two predominant substrates fueling energy supply during exercise (Weber and Haman, 2004). Amino acid metabolism in skeletal muscle might be important for maintaining the metabolic flux of these substrates, via anapleurotic reactions to supply intermediates for the citric acid cycle (Wagenmakers, 1998). However, the direct protein contribution to energy production is minimal in mammals, and often considered negligible (Brooks and Mercier, 1994; Rennie et al., 1981; Wagenmakers, 1998).

The metabolic properties of these fuels play an important role in governing when and where they are used. While lipids have the capacity for highly reduced, anhydrous storage, thereby providing approximately 10-fold more energy per gram wet weight than carbohydrates, their hydrophobic nature means that they require carrier molecules for circulatory and cytoplasmic movement (Hochachka and Somero, 2002; Weber and Haman, 2004). Therefore, lipids comprise a large fraction of total energy stores, primarily as triacylglycerides in adipose tissue, but they can only sustain moderate ATP turnover rates in mammals (Hochachka and Somero, 2002; Weber and Haman, 2004). This makes lipids well-suited for fueling endurance locomotion (Weber and Haman, 2004). Conversely, carbohydrates have many qualities that are important for exercising at a high intensity or under conditions of limited oxygen availability. Carbohydrates yield approximately 15-18% more ATP per mole O_2 than lipids (Brand, 2005), demonstrated *in vivo* in terms of energetic outputs per VO_2 when hummingbirds exclusively utilize each of these fuels (Welch et al., 2007). Furthermore, they can be readily mobilized from intramuscular and hepatic storage depots, and in mammals they are able to sustain maximal ATP turnover rates that are approximately 50% greater than lipids (Hochachka and Somero, 2002; Weber and Haman, 2004).

Lipid oxidation in skeletal muscle is the primary source of energy for low-to-moderate intensities of aerobic exercise (Brooks and Mercier, 1994; Felig and Wahren, 1975; Roberts et al., 1996; Romijn et al., 1993). As exercise intensity increases, absolute rates of both lipid oxidation and carbohydrate metabolism increase, but there is also an escalating reliance on carbohydrate metabolism to supply a greater proportion of total energy (Bergman and Brooks, 1999; Brooks and Mercier, 1994; Edwards et al., 1934; Felig and Wahren, 1975; Roberts et al., 1996; Romijn et al., 1993). Total lipid oxidation rates reach a maximum during moderate-intensity exercise, at approximately 40-60% of the maximal aerobic capacity for exercise (VO_{2max}) in humans; beyond this point there is a decline in absolute lipid flux as well as the proportionate contribution of lipids to energy expenditure (Bergman and Brooks, 1999; Brooks, 1998; Romijn et al., 1993). Approaching VO_{2max}, it is primarily an increasing carbohydrate flux that sustains the rising ATP demand. At this point, carbohydrates are the predominant fuel used within the body, and energy is theoretically supplied exclusively by carbohydrate metabolism at VO_{2max} (Bergman and Brooks, 1999; Brooks and Mercier, 1994; Brooks, 1998; Roberts et al., 1996; Romijn et al., 1993).

The duration of locomotion also affects both the mix of fuels being utilized and the source of fuel substrates. When locomotion is prolonged, circulatory substrates become increasingly more important, relative to intramuscular fuel sources (Felig and Wahren, 1975; Hochachka and Somero, 2002; Romijn et al., 1993). Also, the contribution of lipids to total energy expenditure, particularly the contribution by plasma free fatty acids, gradually increases when exercise at a low-to-moderate intensity is sustained for an extended duration (Felig and Wahren, 1975; Hochachka and Somero, 2002; Romijn et al., 1993).

In general, lipid oxidation in the muscle can draw from both intramuscular triacylglycerol and plasma free fatty acids released from triacylglycerides stored in adipose tissue; carbohydrate metabolism similarly relies on both intramuscular glycogen

stores and circulatory sources of glucose, primarily released from liver glycogen depots (Hochachka and Somero, 2002; McClelland, 2004; Weber et al., 1996a; Weber et al., 1996b; Weber and Haman, 2004). It is not well known how partitioning of these intramuscular and exogenous substrates changes with exercise intensity or duration, but it does seem that with escalating exercise intensity there is an increasing preference for the utilization of intramuscular substrates (particularly glycogen), and the depletion of intramuscular glycogen stores corresponds with the onset of exhaustion or fatigue (Brooks and Mercier, 1994; Felig and Wahren, 1975; McClelland, 2004; Romijn et al., 1993; Weber et al., 1996a; Weber et al., 1996b). Although it has not been widely investigated, there is also inter-species variation in the partitioning of intramuscular and exogenous substrates (McClelland, 2004). For example, compared to goats, dogs appear to place a greater dependence on intramuscular fuel stores, and thus relatively less reliance on circulatory supply (Vock et al., 1996a; Weber et al., 1996a; Weber et al., 1996b).

Conserved aspects of mammalian fuel use patterns

To achieve improved athletic performance, "more aerobic" animals such as dogs (Labrador retrievers, *Canis lupus familiaris*) express biochemical and morphological properties that allow for increased substrate flux through metabolic pathways, compared to less aerobic animals of the same body size, like goats (*Capra hircus*; McClelland, 2004; Roberts et al., 1996; Weber and Haman, 2004). However, this tends to occur in conjunction with an enhanced capacity for delivery and utilization of oxygen, or VO_{2max} (Hochachka and Somero, 2002). Therefore, when the energetic requirements of locomotion are scaled according to the maximal capacity for supporting ATP demand, a specific *absolute* exercise intensity becomes a comparatively lower *relative* exercise intensity (*i.e.* relative to VO_{2max}) in more aerobic individuals (Brooks and Mercier, 1994; Brooks, 1998). When accounting for variation in VO_{2max}, it becomes clear that the relationship between relative exercise intensity and the proportional mix of carbohydrates and lipids fueling energy expenditure appears to be widely conserved among mammalian species examined to date (Fig. 1.1; McClelland, 2004; Roberts et al., 1996; Weber and Haman, 2004).

Furthermore, this conserved relationship has been demonstrated across allometric variation, adaptive variation, and phenotypic plasticity in response to environmental changes. Mass-specific VO_{2max} varies greatly with body mass, but mammals ranging from the size of rats to the size of humans follow a closely conserved pattern, in which relative exercise intensity dictates the proportional mix of lipids and carbohydrates (McClelland, 2004; Roberts et al., 1996; Weber and Haman, 2004). Dogs have evolved with an enhanced ability to perform endurance exercise, and therefore have adaptive alterations that provide an increased total capacity for lipid oxidation, compared to less aerobic animals of a similar body size, like goats (Roberts et al., 1996). However, dogs do not show a shift in fuel preference towards a greater proportional lipid contribution to ATP demand, when compared to goats at any given relative exercise intensity (Roberts et al., 1996). Likewise, animals acclimated to hypobaric hypoxia have a decreased VO_{2max} under hypoxia, and do not shift fuel use towards a higher utilization of carbohydrates

(which provide more ATP yield per mole O_2) compared to animals exercised in normoxia, at least when exercise intensity is expressed relative to VO_{2max} (McClelland et al., 1998; McClelland et al., 1999; McClelland et al., 2001).

Even so, there are some exceptions to this conserved relationship. It seems that in addition to aerobic capacity, variables such as diet, training, and gender can impact fuel use patterns. For instance, mammals can maintain an unchanged VO_{2max} after consuming a low-carbohydrate/high-fat diet for several weeks, despite depleted carbohydrate stores, by increasing capacity for lipid oxidation across a range of relative exercise intensities (Miller et al., 1984; Phinney et al., 1983). Similarly, endurance training can enhance capacity for lipid oxidation, in terms of both the absolute oxidation rates and the proportional contribution to energy supply at low-to-moderate relative exercise intensities (Bergman and Brooks, 1999; Friedlander et al., 1998; Venables et al., 2005). There is also evidence that females exhibit higher maximal rates of lipid oxidation and an increased proportional contribution of lipids to energy expenditure across a range of relative exercise intensities, compared to men (Friedlander et al., 1998; Tarnopolsky et al., 1990; Venables et al., 2005).

Despite the effect of these additional variables, it seems that exercise intensity is the primary determinant of fuel use during exercise (Edwards et al., 1934; Tarnopolsky et al., 1990), and the mammalian fuel use pattern is widely conserved when exercise intensity is scaled to VO_{2max} (McClelland et al., 1998; McClelland, 2004; Weber and Haman, 2004). However, the underlying explanations for this evident unity in fuel use patterns are not currently known. It is also unclear what mechanisms explain the variation between animals in absolute oxidation rates, or if these mechanisms are common across taxa.

Fatty acid uptake and oxidation

Due in part to the complexity of metabolic regulation during exercise, it is not well understood which, if any, of the underlying mechanisms governing whole-body fuel use might be important in maintaining its conserved aspects. For instance, there are myriad points along the fatty acid catabolic pathway which could be targeted for regulation, including free fatty acid mobilization from triacylglycerides, exportation out of adipocytes, circulatory transportation, uptake into myocytes, transport across the mitochondrial membrane, and β-oxidation (Fig. 1.2; Jeukendrup, 2002; McClelland, 2004).

Fatty acids are released from triacylglyceride stores (in both the muscle and adipose tissue) via hydrolysis by hormone-sensitive lipase (McClelland, 2004). In the adipocyte, the hydrophobic free fatty acids are transported through the cytoplasm and across the cell membrane by carrier proteins and transporters, and once released into circulation they travel throughout the body bound to albumin (reviewed in McClelland, 2004).

Upon reaching the muscle, fatty acids cross the endothelium and interstitial space, and are taken into the myocyte itself, although there is some debate surrounding the mechanisms for this uptake. Passive diffusion was long believed to be the primary means for free fatty acids to cross the sarcolemmal membrane, but there is now evidence

suggesting that uptake is largely facilitated by fatty acid transporter proteins, such as fatty acid translocase (FAT/CD36) and plasma-membrane fatty acid binding protein (FABPpm; Glatz et al., 2010; Luiken et al., 1999). Independent inhibition of FAT/CD36 and FABPpm reduces fatty acid uptake by as much as 50%, although the effects are not additive, which supports the possibility that they function in a collaborative manner (Luiken et al., 1999). Furthermore, independent overexpression of several putative fatty acid transporters in rat skeletal muscle stimulates increased fatty acid oxidation in addition to increasing rates of fatty acid transport, and FAT/CD36 was found to be one of the most effective of these transporters in both capacities (Nickerson et al., 2009). These fatty acid transporters have substantial potential for very rapid and localized regulation (Glatz et al., 2010). For instance, muscle contraction induces the translocation of FAT/CD36 from intracellular pools to the cell surface, thus enabling increased rates of fatty acid uptake (Bonen et al., 2000). This is a reversible process, as the transporter is reinternalized after muscle contraction ceases (Bonen et al., 2000).

In the cytoplasm, fatty acids are shuttled intracellularly by cytoplasmic fatty acid binding proteins (FABP; Glatz et al., 2003). The family of intracellular lipid-binding proteins comprises at least nine different tissue-specific types; of these, heart-type FABP (H-FABP) is found within skeletal muscle and is the most abundant cytoplasmic protein therein (Glatz et al., 2003; Paulussen et al., 1989; van Nieuwenhoven et al., 1995). H-FABP performs an important function as an intracellular sink for fatty acids transported into the cytoplasm (Binas et al., 1999; Glatz et al., 2003). However, this role may be permissive rather than limiting to fatty acid uptake under normal conditions (*i.e.* without changing fatty acid transporter content), on account of the abundance of H-FABP protein content in the cytoplasm (Luiken et al., 2003).

Within the myocyte, transportation across the mitochondrial membrane is believed to be another important controlling step in the regulation of fatty acid oxidation, particularly at the level of carnitine palmitoyltransferase (CPT) I in the outer mitochondrial membrane (Jeukendrup, 2002; McClelland, 2004). CPT I converts fatty acyl-CoA (*i.e.* the complex formed by fatty acids within the myocyte) into fatty acyl-carnitine, which can be transferred across the membrane and into the mitochondrial matrix. Inside the inner mitochondrial membrane, CPT II converts fatty acyl-carnitine back into fatty acyl-CoA, which can then enter the β-oxidation spiral. This is a series of reactions catalyzed by such enzymes as medium-chain acetyl-CoA dehydrogenase (MCAD) and β-hydroxyacyl-CoA dehydrogenase (HOAD), ultimately producing acetyl-CoA. Acetyl-CoA is fed into the citric acid cycle at the first step (catalyzed by citrate synthase, CS) and eventually contributes to the production of ATP via oxidative phosphorylation (reviewed in Jeukendrup, 2002; McClelland, 2004).

Mechanisms to control flux through the fatty acid metabolic pathway include translocation of enzymes and transporters within the cell, allosteric or covalent regulation of activity, and modifications to synthesis-degradation pathways (McClelland, 2004). In addition to these mechanisms, chronic regulation of many fatty acid catabolic enzymes and transporters appears to be under some degree of transcriptional control by peroxisome proliferator-activated receptors (PPARs), such as PPARα (McClelland, 2004). PPARα is one of the most predominant isoforms of the PPAR nuclear receptor family in skeletal

muscle, and it is involved in the upregulation of many genes in the fatty acid metabolic pathway (Aoyama et al., 1998; Brandt et al., 1998; Gulick et al., 1994; Zhang et al., 1992). Upon activation, PPARs bind to peroxisome-proliferator-response elements (PPREs) in the promoter regions of these genes (reviewed in McClelland, 2004). Activation of PPARα can occur by the binding of free fatty acids, and this may be one way to regulate the β-oxidation pathway when fatty acids are abundant (McClelland, 2004). Interestingly, there is some implication that members of the FABP family can interact with nuclear receptors, perhaps to transfer long-chain fatty acid ligands as a component of the signaling pathway for fatty acid oxidation (see Schroeder et al., 2008). H-FABP, for instance, may act in concert with PPARα and enhance its transcriptional activity (Tan et al., 2002). Although there is currently little empirical evidence to support H-FABP's proposed role in cytosolic trafficking of fatty acids to nuclear receptors, it remains an intriguing hypothesis for future research.

Carbohydrate utilization

As with the fatty acid metabolic pathway, carbohydrate utilization by muscle involves numerous steps that can be targets for regulation to mediate carbohydrate flux during exercise. One of the most important regulatory points in carbohydrate metabolism appears to be substrate supply to the glycolytic pathway, in the form of exogenous glucose and intramuscular glycogen (Stanley and Connett, 1991).

Unlike fatty acids, carbohydrates are soluble in aqueous fluids and are readily available for sustaining ATP demand during exercise, as they are easily mobilized from glycogen storage depots following the relatively rapid activation of glycogen phosphorylase (Hochachka and Somero, 2002; Stanley and Connett, 1991; Weber and Haman, 2004). Because of this, the main limitations to glucose utilization during exercise seem to occur at the level of transportation across the sarcolemma, or further downstream (Hochachka and Somero, 2002; Rose and Richter, 2005). However, glucose supply, affected by both circulatory perfusion of the muscle and blood glucose concentration, is clearly an important factor for influencing glucose uptake and carbohydrate use (Rose and Richter, 2005).

Skeletal muscle expresses two isoforms of glucose transporters, and of these, insulin-responsive GLUT-4 appears to be the most important regulator of glucose uptake during exercise (Hochachka and Somero, 2002; Rose and Richter, 2005; Stanley and Connett, 1991). It seems that glucose uptake by GLUT-4 is a pivotal limiting factor in the utilization of exogenous glucose, particularly during moderate-intensity exercise (Hochachka and Somero, 2002; Rose and Richter, 2005; Stanley and Connett, 1991). Interestingly, glucose uptake appears to be acutely regulated in a similar manner as transporter-mediated fatty acid uptake: GLUT-4 is translocated from intracellular locations to the plasma membrane when triggered by muscle contraction or insulin stimulation (reviewed in Rose and Richter, 2005; Stanley and Connett, 1991).

Upon entering the myocyte, glucose is phosphorylated to generate glucose-6-phosphate in the initial step of glycolysis. This reaction, catalyzed by hexokinase (HK), is another key regulatory point for determining exogenous carbohydrate utilization during

exercise, as it maintains the downhill glucose gradient from the blood (Halseth et al., 1999; Rose and Richter, 2005). However, it has been suggested that HK is only important as a limiting factor in carbohydrate utilization during high intensity exercise, when build-up of glucose-6-phosphates exerts negative feedback (Rose and Richter, 2005).

As for glycogen, the endogenous source of substrate for the glycolytic pathway, the rate of glycogenolysis is primarily dependent on the activity of glycogen phosphorylase (Stanley and Connett, 1991). This enzyme is subject to multiple levels of regulation, and is therefore an important point for acutely controlling the utilization of intramuscular glycogen during exercise (Stanley and Connett, 1991). Once both glycogen and glucose have been fed into the glycolytic pathway, subsequent regulatory points for determining carbohydrate flux primarily involve key metabolic enzymes, such as phosphofructokinase and pyruvate dehydrogenase (PDH; Spriet and Watt, 2003; Stanley and Connett, 1991).

Regulation of whole-body fuel use

Any of the regulatory points of lipid and carbohydrate muscle metabolism could be potential targets for plastic or adaptive alterations to alter substrate flux through a metabolic pathway and/or adjust the mix of utilized fuels (McClelland, 2004). Therefore, the coordinated regulation of substrate transporter and enzyme activities and amount in the skeletal muscle is likely an important mechanism for mediating whole-body fuel use during exercise (Jeukendrup, 2002; McClelland, 2004). For instance, it has been observed that structural constraints at the level of trans-sarcolemmal transport appear to factor in the greater dependence on intramuscular fuels that occurs with increasing exercise intensity in mammals (Vock et al., 1996b; Weber et al., 1996b).

Substrate availability for metabolism is an obvious factor in the regulation of fuel use in the muscle (McClelland, 2004). Furthermore, it seems that there is interaction between the carbohydrate and lipid metabolic pathways, although the mechanisms are not well understood at present (Spriet and Watt, 2003). Carbohydrate flux appears to directly modulate fatty acid oxidation in skeletal muscle during exercise, potentially by interference at three key regulatory points: fatty acid uptake into the myocyte, lipolysis of intramuscular triacylglycerol, and CPT I activity (Jeukendrup, 2002; Randle et al., 1963; Spriet and Watt, 2003). Likewise, regulation of carbohydrate flux by the fatty acid metabolic pathway may take place at the level of glucose uptake into the myocyte, glycogenolysis, the glycolytic pathway, and PDH activity (Randle et al., 1963; Spriet and Watt, 2003).

Other putative mechanisms regulating fuel use that act on a larger scale include recruitment pattern of muscle fiber types and the endocrine system, specifically changes in norepinephrine, epinephrine, insulin, glucagon, cortisol, and leptin levels (McClelland, 2004). For instance, the increasing proportional reliance on carbohydrates for supplying energy is thought to be partially due to the gradual elevation in sympathetic nervous system stimulation, contraction-induced muscle glycogenolysis, and recruitment of fast glycolytic muscle fibers that occurs with increasing exercise intensity (Brooks and Mercier, 1994). In fact, it has been observed that whole-body fuel use tends to match

progressive muscle fiber type recruitment, and increasing reliance on carbohydrates corresponds with the recruitment of glycolytic muscle fibers (McClelland, 2004; Roberts et al., 1996).

Oxygen availability is another crucial component influencing fuel use, therefore the oxygen cascade plays a role in determining metabolic fuel use during exercise (McClelland, 2004; Weibel et al., 1996). Considering that the mammalian pattern of fuel use appears widely conserved when exercise intensity is scaled to VO_{2max}, it seems likely that determinants of VO_{2max} might be important contributors to fuel use regulation.

Maximal aerobic capacity

Maximal aerobic capacity (VO_{2max}) can be defined as the highest rate at which an animal can take in and utilize oxygen during exercise; this utmost rate of oxygen consumption represents the peak intensity of aerobic exercise (Bassett Jr. and Howley, 2000; Hill and Lupton, 1923). The oxygen cascade consists of a series of steps: 1) ventilation, or intake of oxygenated air into the lungs, 2) diffusion of oxygen into the blood, 3) transport throughout the body via the circulatory system, 4) oxygen diffusion into and within tissues, and lastly 5) utilization at the level of oxidative phosphorylation (Bassett Jr. and Howley, 2000; Hill and Lupton, 1923; Taylor and Weibel, 1981; Wagner, 1996). Oxygen flux (and therefore VO_{2max}) could theoretically be impeded at any of these steps (Bassett Jr. and Howley, 2000; Taylor and Weibel, 1981). Putative central determinants of VO_{2max} include the pulmonary diffusion capacity, maximal cardiac output, and oxygen carrying capacity of the blood (Bassett Jr. and Howley, 2000; Wagner, 1996). Peripheral determinants are primarily skeletal muscle characteristics, such as muscle capillarity and oxidative capacity (Bassett Jr. and Howley, 2000; Wagner, 1996).

Historically, there has been considerable debate as to which step of the oxygen cascade is the most influential in restricting the limits of VO_{2max} in exercising animals. The concept of symmorphosis postulates that structural components of all parts of a system should match overall functional demand, and therefore alterations to enhance flux through all steps of the oxygen cascade would be necessary to increase VO_{2max} (Taylor and Weibel, 1981; Weibel et al., 1991). This hypothesis may be supported by some lines of evidence, such as comparative physiology studies in which most steps of the oxygen cascade were shown to be quantitatively adjusted to match VO_{2max} across a range of allometric and adaptive species variation (Taylor and Weibel, 1981; Weibel et al., 1991). However, while symmorphosis presents an interesting and potentially useful principle for examining physiological systems, it has been widely critiqued as a rigid hypothesis for numerous reasons (Suarez and Darveau, 2005). For instance, it is improbable that forces acting in natural selection impart an "optimal" solution to each biological problem, and furthermore, morphological structures are complex and tend to have multiple, interdependent physiological functions (Dudley and Gans, 1991; Garland and Huey, 1987).

In fact, while it *is* unlikely that there is a single limiting factor for VO_{2max}, it seems that each step in the oxygen cascade has an integrated effect, and the relative contribution of each step varies, depending on physiological conditions (Bassett Jr. and

Howley, 2000; Suarez and Darveau, 2005; Wagner, 1996). For example, under hyperoxic conditions there can be an elevation in VO_{2max}, but this effect may only be significant in highly trained individuals (Powers et al., 1989). This suggests that with increased oxygen availability, the ability to increase flux through the oxygen cascade depends on physiological variables such as training status, and untrained individuals may be differentially limited at some step in the oxygen transport pathway. In general, it seems that central factors influencing oxygen supply to the muscle – primarily those related to cardiac output – are the dominant constraints of VO_{2max}, at least in humans under normal conditions (Bassett Jr. and Howley, 2000; Wagner, 1996).

As oxygen is the terminal electron acceptor in oxidative phosphorylation, oxygen availability is an important factor in regulating fuel use and substrate selection in skeletal muscle; conversely, ATP demand has a major role in dictating oxygen intake and delivery (Hochachka and Somero, 2002). Furthermore, many of the steps in the oxygen cascade that constrain VO_{2max} are also influential in the regulation of whole-body fuel use. For instance, such cardiovascular factors as cardiac output and muscle capillarity influence the delivery of oxygen *and* circulatory fuels to the muscle, as both share a convective supply pathway. In addition, muscle oxidative capacity, with respect to maximal activities of metabolic enzymes for instance, is a peripheral determinant of VO_{2max}. Given the integrated nature of the oxygen transport pathway and metabolic fuel use, it is perhaps unsurprising that absolute rates of fuel use are scaled with differences in aerobic capacity in mammals.

Thus, it seems probable that an underlying explanation for the conserved mammalian fuel use pattern might lie within some matched, interdependent aspect of these two systems. Selective breeding experiments allow for an examination of complex physiological traits without the complications of phylogenetic diversity, in contrast to most of the comparative approaches that use diverse species. In light of this, a model of experimental evolution which features innate differences in locomotor activity and maximal aerobic capacity could be useful for an investigation of fuel use patterns and regulatory mechanisms of metabolic flux.

Selectively bred high-running mice

An experimental evolution project was started in 1993, to develop a selectively bred mouse model that exhibits high levels of voluntary wheel-running (see Table 1.1). Voluntary wheel-running may be an ecologically relevant indicator of locomotor activity in the wild, and rodents engage in it to the extent that it is physically demanding exercise which elicits morphological and physiological changes (Garland, 2003; Swallow et al., 1998a). Furthermore, the large amount of individual variation in voluntary locomotion seems to be heritable, to some degree, and may have been a selectively-important trait in the past (Swallow et al., 1998a). Natural selection is based to some degree upon ecological performance (*i.e.* how organisms choose to utilize performance abilities), as opposed to solely operating on maximal capacity for performance (Husak, 2006).

This selective breeding project has been described in detail by Swallow and colleagues (Swallow et al., 1998a). In brief, a founder population of outbred, genetically variable house mice (Hsd:ICR strain) was randomly mated for two generations. Ten pairs

were then assigned to each of eight closed lines: four selectively bred "high running" (HR) lines, and four non-selected control lines. When mice reach 6-8 weeks of age, they are individually housed with continuous access to activity wheels for six consecutive days, during which time the number of wheel revolutions per minute is measured. Selection is based on the total number of revolutions on days five and six. In the HR lines, the highest running male and female from each family are selected as breeders. In control lines, one male and one female from each family are randomly chosen to be breeders. In all cases, sibling mating is disallowed.

By generation ten of selection, mice from the HR lines ran on average 75% more than control mice (Swallow et al., 1998a). Since generation 16, divergence in locomotion seems to have reached a relatively stable plateau, with HR mice exhibiting approximately 170% more voluntary wheel-running than control mice (Fig. 1.3; Garland, 2003). This range in voluntary locomotion is comparable to the range across 13 species of wild murid rodents, which suggests that the divergence between HR and control lines is ecologically and evolutionarily relevant (Dewsbury, 1980; Garland, 2003). Furthermore, HR mice demonstrate a significantly increased capacity for endurance locomotion, compared to control mice (Meek et al., 2009). Given that lipids are an important fuel for endurance-type exercise, it is likely that HR mice will therefore have an enhanced capacity for lipid oxidation.

HR mice appear to primarily achieve greater levels of locomotor activity (*i.e.* increased wheel revolutions per day) by means of increased running speeds, rather than more time spent running (Garland, 2003; Garland et al., 2010; Girard et al., 2001; Swallow et al., 1998a). However, there is some component of sex-specific response in locomotor behavior, as HR males spend more time running than control males, in addition to running at higher speeds; HR females simply run faster and more intermittently than control females (Garland et al., 2010; Girard et al., 2001; Rezende et al., 2009).

Selection for high locomotor activity has also led to an increased aerobic capacity in HR lines, particularly amongst male mice (Rezende et al., 2006; Rezende et al., 2006b; Swallow et al., 1998b). Moreover, the physiological constraints of VO_{2max} appear to differ between control and HR mice, which suggests that selection for high locomotor activity has affected some components of the oxygen transport pathway (Rezende et al., 2006). It seems that while both peripheral and central factors play a role in restricting VO_{2max} for all mice, control mice might be limited to a greater degree by central factors in the oxygen cascade (Rezende et al., 2006a; Rezende et al., 2006).

However, HR mice do not differ from control mice in a number of subordinate traits that are potentially important for oxygen transport or aerobic metabolism (Table 1.1). There does not appear to be significant differences in aerobic capacity of cardiac or skeletal muscles, at least with respect to myoglobin concentration or maximal CS activity (Rezende et al., 2006a). Furthermore, HR and control mice do not differ in the oxygen carrying capacity of the blood, in terms of haematocrit, blood hemoglobin content, or the affinity of blood hemoglobin for oxygen (Rezende et al., 2006a; Swallow et al., 2005). However, with continuous access to running wheels, the training-induced elevation in hematocrit and blood hemoglobin content is significantly augmented in the HR lines

(Swallow et al., 2005). Notably, this does not appear to be entirely a function of the increased amount of wheel-running by HR mice during this "training period." Rather, it is an example of enhanced phenotypic plasticity, which is the ability to adjust phenotype in response to changed conditions (Garland and Kelly, 2006).

This is reflective of another component of selective breeding experiments. Artificial selection favors a particular trait, but it can also favor alleles that increase phenotypic plasticity towards the trait in question (Garland and Kelly, 2006). In other words, since the selective regime for these HR mice is based on the amount of voluntary locomotion on the fifth and sixth days of wheel access, it may be inadvertently selecting for the ability to enhance the training effects of wheel access (*i.e.* increased phenotypic plasticity), in addition to selecting for innate or instantaneous traits that impact locomotion (Garland and Kelly, 2006).

In this manner, it appears that HR mice have a greater plasticity or capacity for adjusting hematocrit and blood hemoglobin content with exercise training, but no innate differences in these traits without exercise training (Garland and Kelly, 2006; Swallow et al., 2005). This also appears to be the case for the aerobic enzymes PDH and cytochrome c oxidase (CCO), where training HR females show a greater capacity to increase the maximal activities of these enzymes in mixed hindlimb muscle, compared to control mice (Garland and Kelly, 2006; Houle-Leroy et al., 2000). Similarly, with access to activity wheels, HR mice also have augmented protein levels of the glucose transporter GLUT-4 in the gastrocnemius, relative to control mice (Gomes et al., 2009).

The "mini muscle" phenotype

Selection for high levels of locomotion has resulted in a number of additional physiological modifications in the HR lines that are independent of training (Table 1.1). For instance, adult HR mice are significantly smaller in total body mass and have reduced body fat content, compared to control mice (Dumke et al., 2001; Swallow et al., 1999). But perhaps the most unexpected physiological change that has appeared in the HR lines is the "mini muscle" phenotype, caused by a single autosomal recessive allele (Garland et al., 2002).

The emergence of this phenotype was evident as early as the sixth generation of selection, with increasing frequency in two of the four HR lines that became significant by generation 22 (Fig. 1.4; Garland et al., 2002). In the other two HR lines, this allele was presumably lost due to random genetic drift (Garland et al., 2002). In mice with this unique phenotype, the mass of the triceps surae complex – comprised of the gastrocnemius, plantaris, and soleus muscles – of "mini muscle" (HR_{mini}) mice is halved, compared to the normal phenotype (Garland et al., 2002). The "mini muscle" phenotype is characterized by this reduction in hindlimb muscle mass, coupled with increased mass-specific aerobic capacity of the muscles (Table 1.1; Garland et al., 2002; Houle-Leroy et al., 2003).

By generation 35, HR_{mini} mice were demonstrating even greater levels of voluntary locomotion than selected HR mice with the normal muscle phenotype (HR_{normal} mice), predominately due to increased running speeds (Gomes et al., 2009; Syme et al., 2005). Moreover, female HR_{mini} mice were able to achieve a higher VO_{2max} than HR_{normal}

mice at generation 36, but only under hypoxic conditions (Rezende et al., 2006). It was proposed that increased cardiac output due to heart enlargement in HR_{mini} mice could contribute to this improved aerobic performance under hypoxia (Rezende et al., 2006a; Rezende et al., 2006).

The reduction in "mini muscle" size is largely compensated by an increase in mass-specific aerobic capacity, evidenced by substantially enhanced mass-specific activities of HK, total PDH, and the mitochondrial enzymes CPT II, CS, and CCO (Bilodeau et al., 2009; Guderley et al., 2006; Guderley et al., 2008; Houle-Leroy et al., 2003; Rezende et al., 2006a). There also seems to be a simultaneous reduction in the mass-specific anaerobic capacity of these muscles, indicated by lowered maximal activity of lactate dehydrogenase, as well as a reduced whole-muscle glycogen phosphorylase content (both the activated form and total content; Guderley et al., 2006; Guderley et al., 2008; Houle-Leroy et al., 2003).

The primary physical basis for differences in "mini muscle" size and metabolic properties appears to be a loss or dramatic reduction of type IIB (fast glycolytic) fibers in many of the hindlimb muscles (Bilodeau et al., 2009; Guderley et al., 2006; McGillivray et al., 2009). The reduced proportion of type IIB fibers corresponds with the appearance of small, minimally differentiated fibers or atypical disordered regions in the gastrocnemius and plantaris (Bilodeau et al., 2009; Guderley et al., 2006; Guderley et al., 2008). Furthermore, it occurs in conjunction to an increased proportion and fiber size of the oxidative type I and IIA fibers, a higher mitochondrial volume density in the muscle, and increased mass-specific myoglobin content (Bilodeau et al., 2009; Guderley et al., 2006; Guderley et al., 2008; Rezende et al., 2006a).

In fact, hindlimb muscles with a normally higher proportion of type IIB fibers such as the gastrocnemius, show the greatest reduction in mass; the soleus, which is normally primarily composed of type I and type IIA fibers, actually shows a relative enlargement in the HR_{mini} mice (Guderley et al., 2006; Syme et al., 2005). Mechanical performance of the altered gastrocnemius suggests that both the speed of contraction and the absolute power potential is reduced, and that it is more resistant to fatigue (Syme et al., 2005). Together, these relative changes in sizes of "slow-twitch" and "fast-twitch" muscles would theoretically increase endurance capacity of HR_{mini} mice (Syme et al., 2005).

However, altered "mini muscles" do not have increased mechanical efficiency (McGillivray et al., 2009), and HR_{mini} mice actually appear to exhibit an increased whole-body energetic cost of transport, compared to HR_{normal} mice (Dlugosz et al., 2009). This suggests that some aspect other than physical muscle size confers the selective benefit of this unusual phenotype (Dlugosz et al., 2009). For instance, a higher capillary-to-fiber ratio and higher capillary density in the HR_{mini} mouse hindlimb muscles likely contributes to their superior mass-specific aerobic function (Wong et al., 2009). Moreover, HR_{mini} females without prior wheel access also have higher mass-specific glycogen stores in the gastrocnemius and the liver, compared to mice with the normal muscle phenotype (Gomes et al., 2009).

Interestingly, many aspects of the "mini muscle" phenotype (Table 1.1) are consistent with the changes that occur with aerobic exercise training (Houle-Leroy et al.,

2003). These include increased intramuscular content of fuel substrates, increased levels of key enzymes in aerobic metabolic pathways, reduced activity levels of anaerobic enzymes concurrent to elevated aerobic capacity, and increased skeletal muscle capillarity (Hochachka and Somero, 2002). Furthermore, interspecies comparisons reveal that an increased proportion of more oxidative fiber types in the skeletal muscle is correlated with increased aerobic performance (Hochachka and Somero, 2002).

On the other hand, skeletal muscle hypertrophy would theoretically enhance whole-body aerobic performance (Hochachka and Somero, 2002), but HR_{mini} mice show quite the opposite characteristic. Nonetheless, considering that endurance training appears to increase the proportional contribution of lipids to energy supply at low-to-moderate relative exercise intensities (Bergman and Brooks, 1999; Venables et al., 2005), it seems likely that HR_{mini} mice might exhibit a similar shift in the whole-body metabolic fuel use pattern during exercise.

Objectives

The aim of my investigation was to use this model of experimental evolution to determine if whole-body metabolic fuel use during exercise can be adaptively altered or differentially regulated if animals are under selection for high locomotor activity. To fulfill this goal, my specific objectives were:

1) To assess VO_{2max} in an HR_{mini} line (in which the "mini muscle" phenotype has become fixed), an HR_{normal} line (in which the "mini muscle" allele has been lost), and a non-selected control line.

2) To determine whole-body fuel use across a range of submaximal relative exercise intensities in HR_{mini}, HR_{normal}, and control mice.

3) To characterize some physical and metabolic cardiac properties in these mice.

4) To evaluate physiological factors involved in the regulation of skeletal muscle fuel use, specifically: intramuscular glycogen stores; muscle recruitment patterns (as represented by exercise-induced glycogen depletion); maximal activity levels of the metabolic enzymes CPT II, HOAD, CS, and HK; mRNA content of *FAT/CD36*; cytosolic protein content of H-FABP; and mRNA levels of several transcriptional factors involved in mediating metabolic pathways, including PPARα.

In this investigation, I tested the following hypotheses:

Hypothesis 1) HR_{mini} mice will have an enhanced whole-body ability to oxidize lipids, and will exhibit a shift in the fuel use pattern towards the utilization of a greater proportion of lipids than HR_{normal} or control mice at any given relative exercise intensity.

Hypothesis 2) If the fuel use pattern is conserved amongst all mice, the two phenotypes of HR mice (HR_{mini} and HR_{normal}) will diverge at some regulatory points in substrate uptake and metabolic pathways, thus achieving a common pattern of fuel use via differential mechanisms.

Figure 1.1: The relationship between relative exercise intensity and the proportionate mix of lipids and carbohydrates supplying total ATP demand in mammals.

The relationship ($r^2 = 0.83$, not including altitude data) between relative exercise intensity ($\%VO_{2max}$) and percentage of total fuel oxidation ($\%VO_2$) supplied by (A) lipids and (B) carbohydrates (CHO) in animals of the same (dogs and goats) or different (rats and humans) sizes but all with different aerobic capacities. Data are from dogs and goats; trained and untrained rats; trained men; trained and untrained women; and altitude acclimated (HA) and sea level (SL) rats. Figure reproduced from McClelland (2004).

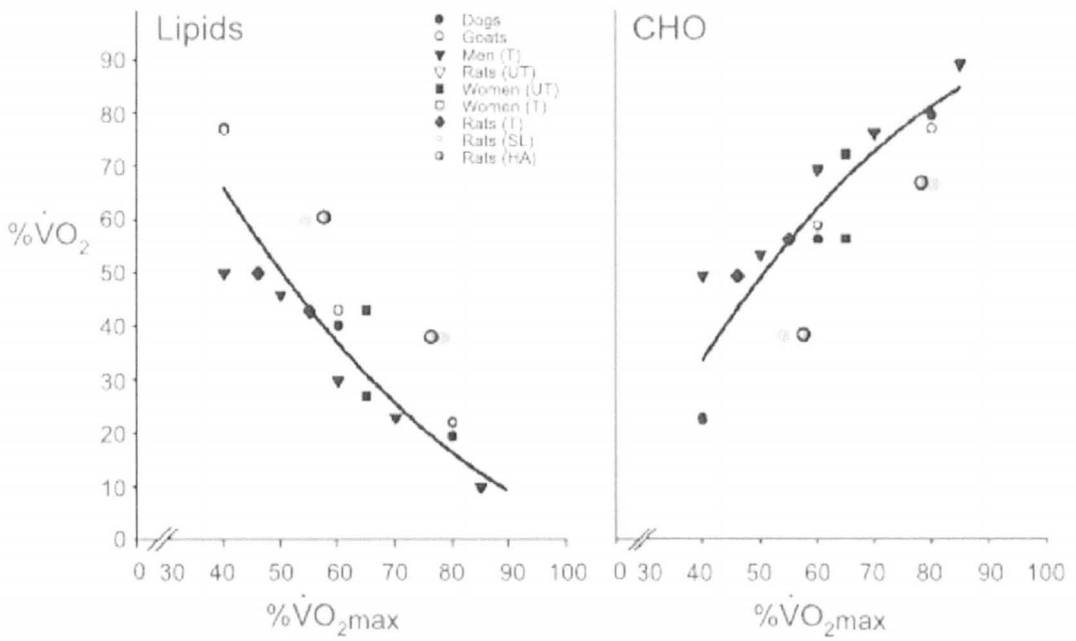

Lipids
CHO
Dogs
Goats
Men (T)
Rats (UT)
Women (UT)
Women (T)
Rats (T)
Rats (SL)
Rats (HA)
%V̇O2
%V̇O2max
%V̇O2max
0 30 40 50 60 70 80 90 100
0 30 40 50 60 70 80 90 100
0 10 20 30 40 50 60 70 80 90

Figure 1.2: Pathway of fatty acid mobilization, uptake, and oxidation.

As fatty acids (filled circles) move from storage sites to muscle mitochondria to provide ATP for contraction, they must be mobilized (M) from storage forms, pass through specific membrane transporters (TR), and undergo either circulatory or cytosol transport (T) steps. Entry into the mitochondria is through specific transporters, and free fatty acids undergo β-oxidation (β-ox) with each pass through the reaction spiral that yields acetyl-CoA. Acetyl-CoA then enters the tricarboxylic acid cycle (TCA) or citric acid cycle, producing reducing equivalents for the production of ATP and the subsequent generation of CO2. Figure reproduced from McClelland (2004).

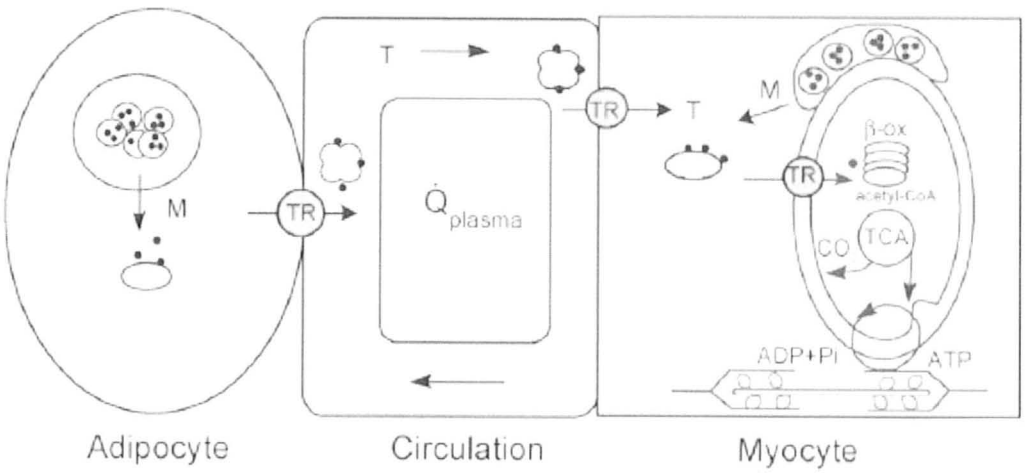

T
M
TR
$\dot{Q}_{plasma}$
TR
T
M
β-ox
acetyl-CoA
TR
CO
TCA
ADP+Pi
ATP
Adipocyte
Circulation
Myocyte

Table 1.1: Overview of behavioral and physiological changes that emerged in selectively bred high running mice.

↑ or ↓ signifies significant difference compared to non-selected control mice (in "HR mice" column) or compared to normal muscle phenotype (in "Mini muscle" column). A dash (-) indicates that no significant effect was detected.

Trait		HR mice	"Mini muscle"	Comments	References
Locomotor behavior and capacity	Voluntary locomotion	↑ (~1.8x)	-	Detected at 10th generation	Swallow et al., 1998a
		↑ (~2.6x)	↑	Plateau by 16th generation; Effect of "mini muscle" detected at 35th generation	Garland, 2003; Gomes et al., 2009; Syme et al., 2005
	Running speed	↑	↑	Detected at 10th generation; At 23rd generation, more intermittent running in HR females also detected	Girard et al., 2001; Gomes et al., 2009; Swallow et al., 1998a; Syme et al., 2005
	Time spent running	↑ (males only)	-	Trend detected at 10th generation; Statistical significance at 32nd generation	Garland, 2003; Garland et al., 2010; Rezende et al., 2009; Swallow et al., 1998a
	Endurance capacity	↑	-	Detected at 49th generation	Meek et al., 2009
	Aerobic capacity (VO_{2max})	↑ (6-18%)	↑ (only with hypoxia)	Detected at 10th generation; Effect of "mini muscle" (hypoxia) detected at 36th generation	Rezende et al., 2006; Rezende et al., 2006b; Swallow et al., 1998b
	Cost of transport	-	↓	Detected at 46th generation	Dlugosz et al., 2009
Cardiac properties	Cardiac myoglobin	-	-	At 36th generation	Rezende et al., 2006a
	Cardiac CS activity	-	-	At 36th generation	Rezende et al., 2006a
	Relative heart size	-	↑	Detected at 36th generation	Rezende et al., 2006a

Blood O_2 carrying capacity	Hct, hemoglobin *Sedentary mice*	-	-	At 36th generation	Swallow et al., 2005
	Hct, hemoglobin *With wheel access*	↑	-	Detected at 14th generation	Swallow et al., 2005
Hindlimb muscle: physical properties and fiber types	Muscle mass	*see comments*	↓ (~0.5x)	Increasing frequency of "mini muscle" in generations 6-22	Garland et al., 2002
	Proportion of type IIB muscle fibers	-	↓ (~0x)	Detected at 26th generation	Bilodeau et al., 2009; Guderley et al., 2006; Guderley et al., 2008;
	Capillarity	-	↑	Detected at 36th generation	Wong et al., 2009
	Myoglobin content	-	↑	Detected at 36th generation	Rezende et al., 2006a
	Mechanical efficiency	-	-	At 46th generation	McGillivray et al., 2009
Hindlimb muscle: metabolic properties	PDH, CCO (Ug^{-1}) *Sedentary mice*	-	↑	Detected at 14th generation	Houle-Leroy et al., 2000; Houle-Leroy et al., 2003
	PDH, CCO (Ug^{-1}) *With wheel access*	↑	-	Detected at 14th generation	Houle-Leroy et al., 2000
	HK, CPT II, CS (Ug^{-1})	-	↑ (~2x)	Detected at 14th generation	Guderley et al., 2006; Guderley et al., 2008; Houle-Leroy et al., 2003
	LDH (Ug^{-1})	-	↓	Detected at 14th generation	Guderley et al., 2006; Guderley et al., 2008; Houle-Leroy et al., 2003
	Whole-muscle glycogen phosphorylase	-	↓	Detected at 14th generation	Guderley et al., 2006; Guderley et al., 2008; Houle-Leroy et al., 2003
	Glycogen content	-	↑	Detected at 35th generation	Gomes et al., 2009
	GLUT-4 protein *Sedentary mice*	-	-	At 35th generation	Gomes et al., 2009
	GLUT-4 protein *With wheel access*	↑	-	Detected at 35th generation	Gomes et al., 2009

Figure 1.3: Voluntary locomotion in male mice from selected HR lines and non-selected control lines

Wheel running (line means) of eight lines of house mice either selected for high wheel running or bred randomly as controls. Dips in wheel running that occur approximately every four generations correspond to summer generations, during which elevated humidity (and sometimes temperature) may reduce activity. Figure reproduced from Garland (2003).

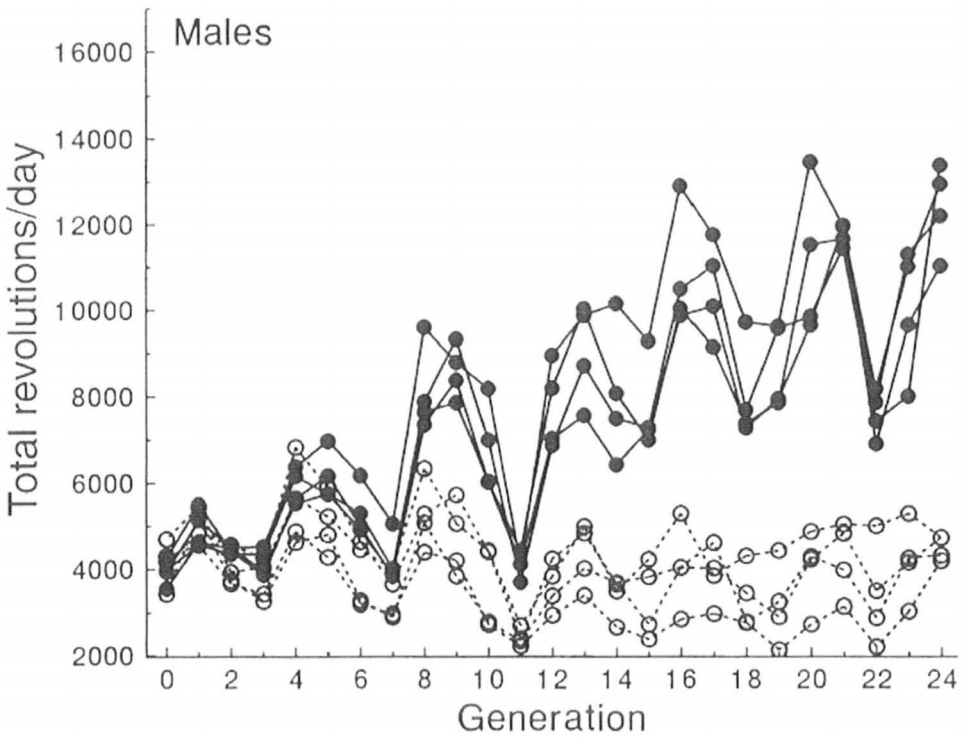
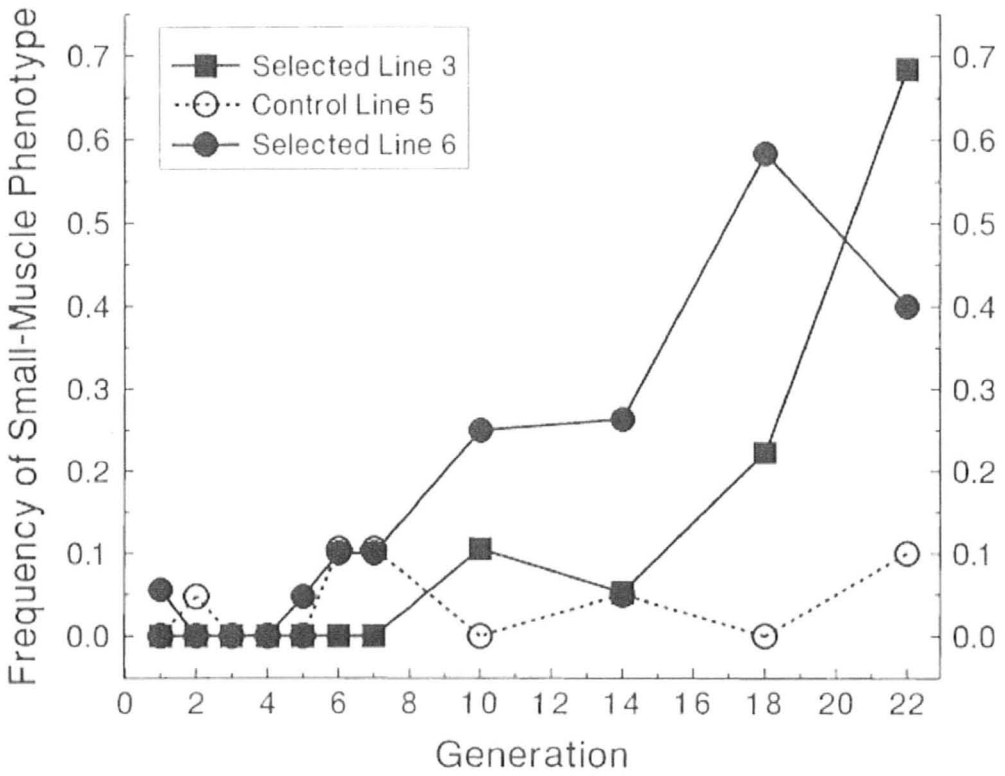

Males
Total revolutions/day
16000
14000
12000
10000
8000
6000
4000
2000
0
2
4
6
8
10
12
14
16
18
20
22
24
Generation

Figure 1.4: Emergence of "mini muscle" phenotype in response to selective breeding for high locomotor activity.

Frequency of small-muscle phenotype in selected and control treatments over 22 generations (sample size is 10 males and 10 females per line for each generation, with the exception of occasional missing data). Phenotype was never observed in two of the selected and in three of the control lines. Figure reproduced from Garland et al. (2002).


Frequency of Small-Muscle Phenotype
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Selected Line 3
Control Line 5
Selected Line 6
0
2
4
6
8
10
12
14
16
18
20
22
Generation

CHAPTER 2: DIVERSITY IN THE REGULATION OF METABOLIC FUEL USE IN MICE SELECTED FOR HIGH LOCOMOTOR ACTIVITY

Abstract

Despite diversity in locomotion, the mammalian pattern of metabolic fuel use appears highly conserved, with relative exercise intensity dictating the proportionate contribution of carbohydrates and fats to energy supply. To determine if selection for high locomotion can change this fuel use pattern or its regulation, I used mice selectively bred for high wheel running (HR mice). Within this model, a "mini muscle" phenotype of half-sized hindlimb muscles with increased mass-specific aerobic capacity has emerged in two of four HR lines. I used indirect calorimetry to assess fuel use during exercise, in one line each of: 1) non-selected controls, 2) HR "mini-muscle" (HR_{mini}) mice, and 3) HR mice with the normal muscle phenotype (HR_{normal} mice). I also evaluated cardiac properties and factors that regulate skeletal muscle fuel use.

HR mice had an increased VO_{2max}, compared to control mice, and VO_{2max} was also higher in HR_{mini} than HR_{normal} mice. A greater relative heart mass in HR_{mini} mice and cardiac metabolic remodeling in both HR lines may support their enhanced VO_{2max}. However, there were no significant differences in the pattern of proportionate fuel use across a range of relative exercise intensities for HR_{mini}, HR_{normal}, and control mice. In HR mice, absolute lipid oxidation rates were greater at moderate exercise intensities, commensurate with elevations in VO_{2max}.

HR_{mini} mice and HR_{normal} mice appear to have alternate means of increasing lipid oxidation capacity. HR_{mini} mice have increased skeletal muscle cytosolic fatty acid binding protein (H-FABP), fatty acid transporter (*FAT/CD36*) mRNA, peroxisome proliferator-activated receptor (*PPAR*) α mRNA, and mass-specific activities of aerobic enzymes, as well as a different pattern of muscle recruitment during exercise. Conversely, HR_{normal} mice have greater whole-muscle metabolic enzyme activities due to muscle enlargement. Therefore, mammals can differentially utilize mechanisms for enhancing fuel oxidation rates, without deviating from a conserved pattern of fuel use.

Introduction

A major component of maintaining the essential balance between ATP production and ATP demand when there is increased energy demand, such as during exercise, is the ability to both select an appropriate mixture of metabolic fuels and adjust total flux of substrates through metabolic pathways (Hochachka and Somero, 2002; Weber and Haman, 2004). In exercising mammals, the two predominant substrates sustaining energy expenditure in the muscles are lipids (drawing from intramuscular triacylglycerides and plasma free fatty acids) and carbohydrates (sources of intramuscular glycogen depots and blood glucose; Weber and Haman, 2004). The synthesis of required intermediates for the citric acid cycle, a component of amino acid metabolism in muscle, may be important for supporting substrate flux through metabolic pathways, but the direct protein contribution to energy supply is minimal in mammals (Brooks and Mercier, 1994; Wagenmakers, 1998).

Low-to-moderate intensity exercise is primarily fueled by the oxidation of lipids, which constitute a large fraction of energy reserves in mammals due to their concentrated, highly reduced storage form (mainly as triacylglycerides stored in adipose tissue; Brooks and Mercier, 1994; Felig and Wahren, 1975; Romijn et al., 1993; Weber and Haman, 2004). As exercise intensity increases, the proportion of energy expenditure supported by carbohydrates escalates, partially due to a gradual elevation in contraction-induced muscle glycogenolysis, sympathetic nervous system stimulation, and recruitment of fast glycolytic muscle fibers (Brooks and Mercier, 1994; Edwards et al., 1934; Felig and Wahren, 1975). Approaching the maximal aerobic capacity (VO_{2max}), lipid flux and the lipid contribution to ATP demand declines, while carbohydrates – capable of sustaining a high ATP turnover rate, with a greater yield of ATP per mole O_2 than lipids (Brand, 2005; Welch et al., 2007) – become the predominant fuel (Brooks and Mercier, 1994; Brooks, 1998; Roberts et al., 1996; Romijn et al., 1993; Weber and Haman, 2004). Theoretically, carbohydrates are the sole suppliers of energy at VO_{2max}.

Species that are adaptively "more aerobic" tend to have a greater capacity for total substrate flux through metabolic pathways than less aerobic species. For example, across a range of submaximal exercise intensities, lipid and carbohydrate oxidation rates in dogs are approximately double the rates achieved by goats, although these are similarly sized animals (Roberts et al., 1996). The same can be said of mammals ranging in body size, with smaller mammals exhibiting greater mass-specific rates of fuel oxidation, in conjunction with their enhanced mass-specific aerobic capacity (Weber and Haman, 2004).

However, if variation in VO_{2max} is accounted for by assessing *relative* exercise intensity (*i.e.* relative to VO_{2max}), the relationship between intensity and the proportional mix of lipids and carbohydrates contributing to energy supply is closely conserved amongst a wide range of mammalian species, irrespective of allometric variation or diversity in locomotor capabilities (McClelland, 2004; Roberts et al., 1996; Weber and Haman, 2004). Furthermore, this relationship may withstand modifications to aerobic capacity, such as those ensuing from acclimation to low O_2 (McClelland et al., 1998;

McClelland et al., 1999; McClelland et al., 2001). Even variability in the source of the fuel substrates utilized by working muscle does not tend to alter the proportionate mix of carbohydrates and lipids. For instance, dogs appear to place a greater dependence on intramuscular glycogen and triacylglyceride stores (and are correspondingly less reliant on circulatory fuel supply) compared to goats, but both species utilize an equivalent mix of carbohydrates and lipids at a given relative exercise intensity (Roberts et al., 1996; Vock et al., 1996a; Weber et al., 1996a; Weber et al., 1996b).

Despite the conserved nature of this relationship, there are some exceptions. It seems that in addition to aerobic capacity, variables such as diet, training status, and gender can impact fuel use patterns. Specifically, females (Friedlander et al., 1998; Tarnopolsky et al., 1990; Venables et al., 2005) as well as individuals that have either been endurance-trained (Bergman and Brooks, 1999; Friedlander et al., 1998; Venables et al., 2005) or maintained on a low-carbohydrate/high-fat diet (Miller et al., 1984; Phinney et al., 1983) all exhibit an enhanced capacity for lipid oxidation and an increased proportional contribution of lipids to energy expenditure across a range of relative exercise intensities, in comparison to their counterparts. Even so, the relationship between relative exercise intensity and the proportionate mix of carbohydrates and lipids fueling exercise in mammals is surprisingly robust and conserved (McClelland, 2004; Weber and Haman, 2004). An animal model which features innate variation in the complex trait of voluntary locomotion might reveal some of the underlying means by which genetically and phenotypically distinct mammals retain a conserved pattern of fuel use, or alternately, what might contribute to deviations from that conserved pattern.

An experimental evolution project was begun in 1993 to develop a mouse model selectively bred for high levels of voluntary wheel-running (see Swallow et al., 1998a for details). In brief, there are eight closed lines in this project: four "high running" (HR) lines in which there is selective breeding for high levels of locomotion, and four non-selected control lines. When the mice are 6-8 weeks old, they are individually housed with continuous access to activity wheels for six days; selection in the HR lines is based on the total number of wheel revolutions on the fifth and sixth days (Swallow et al., 1998a). By generation 16 of selection, HR lines were running on average 170% more than control lines, although they seem to have reached a plateau in voluntary locomotion by this point (Garland, 2003).

A major strength of this model was the incorporation of multiple replicates for both HR lines and non-selected control lines in the experimental design. This provided opportunities for the development of "multiple solutions" (*i.e.* differential adaptive changes in subordinate traits) in response to a uniform selective influence (Garland, 2003). For instance, a "mini muscle" phenotype – characterized by half-sized hindlimb muscles with a doubling of mass-specific aerobic capacity, caused by a single autosomal recessive allele – has emerged in two of the HR lines (Garland et al., 2002; Garland, 2003). By generation 35, HR mice with this "mini muscle" phenotype (HR_{mini} mice) were exhibiting even greater levels of voluntary running than selected HR mice with the "normal" muscle phenotype, referred to as HR_{normal} mice (Gomes et al., 2009; Syme et al., 2005).

The primary physical basis for the distinctive skeletal muscle properties of HR_{mini} mice appears to be a drastic reduction in type IIB (fast glycolytic) muscle fibers (Bilodeau et al., 2009; Guderley et al., 2006). This reduction corresponds with an increased fiber size and proportion of the oxidative type I and IIA muscle fibers and a higher mitochondrial volume density in hindlimb muscles (Bilodeau et al., 2009; Guderley et al., 2006; Guderley et al., 2008). In addition, this has led to increased mass-specific activities of aerobic enzymes and reduced mass-specific activities of anaerobic enzymes (Bilodeau et al., 2009; Guderley et al., 2006; Guderley et al., 2008; Houle-Leroy et al., 2003). In light of these alterations, it is particularly notable that progressive recruitment of type IIB muscle fibers with increasing exercise intensity is thought to be instrumental in influencing the gradual shift of fuel preference towards carbohydrates; coordinated regulation of enzyme activities also plays a role in adjusting flux through a metabolic pathway (Brooks, 1998; McClelland et al., 1998; McClelland, 2004; Roberts et al., 1996). It is therefore possible that HR_{mini} mice, exhibiting many traits that are characteristic of endurance-trained mammals, may show a shift in their fuel use pattern.

In addition to the emergence of the "mini muscle" phenotype, selection for high locomotor activity also led to an increased aerobic capacity (VO_{2max}) in HR lines, particularly amongst males (Rezende et al., 2006; Rezende et al., 2006b; Swallow et al., 1998b). Furthermore, female HR_{mini} mice can achieve a greater VO_{2max} than HR_{normal} mice, but only under hypoxic conditions (Rezende et al., 2006). It was proposed that heart enlargement in HR_{mini} mice could contribute to this increased aerobic performance under hypoxia, which is interesting because cardiac output centrally restricts VO_{2max}, and there is some indication that the aerobic capacity of HR mice is limited to a lesser degree by central determinants in the O_2 cascade (*i.e.* factors affecting oxygen delivery to the muscle), compared to control mice (Rezende et al., 2006a; Rezende et al., 2006). However, beyond the evidence that HR mouse hearts do not have enhanced citrate synthase (CS) activity or myoglobin content (Rezende et al., 2006a), there has been limited characterization of the hearts of these mice, and it has not yet been ascertained if they show significant physical (*i.e.* hypertrophic) or metabolic remodeling.

Based on the differences observed between HR and control lines, it seemed that there would also be changes to fuel use during exercise. I predicted that HR mice – HR_{mini} mice in particular, considering the increased mass-specific muscle aerobic capacity and reduction in type IIB muscle fibers – would have an enhanced capacity for lipid oxidation to support their sustained aerobic exercise. However, given to the robustness of the mammalian fuel use pattern, it also seemed likely that this increased lipid oxidation would scale with changes in aerobic capacity, resulting in a similar mix of fuels supporting energy expenditure for both HR and control mice, across a range of relative exercise intensities. If that were the case, I predicted that there would be differences between HR_{mini} mice and HR_{normal} mice at specific steps in substrate delivery and metabolic pathways, considering the divergence in skeletal muscle phenotypes.

Although the mechanistic explanation for the unity of fuel use patterns in mammals has not been fully delineated, the proportionate contribution of carbohydrates and lipids to energy supply is likely influenced by many factors, including recruitment of different muscle fiber types, the endocrine system, substrate and oxygen availability, and

the regulation of enzyme and substrate transporter quantities and activities (Brooks, 1998; McClelland et al., 1998; McClelland, 2004). In my study, I examined several contributing regulators of whole-body fuel use in HR_{mini}, HR_{normal}, and control mice, namely: intramuscular glycogen stores, muscle recruitment patterns, maximal activity levels of metabolic enzymes, mRNA and protein content of fatty acid transporters and carriers, and mRNA levels of transcriptional factors involved in mediating metabolic pathways. The fatty acid metabolic pathway was the main focus of this investigation; it seemed likely there would be changes in this pathway resulting in an increased fatty acid oxidative capacity in HR mice. As the gastrocnemius is a major hindlimb muscle in rodents, with a normally high proportion of type IIB fibers (Armstrong and Phelps, 1984; Hamalainen and Pette, 1993) – and therefore striking alterations in muscle size and properties in HR_{mini} mice (Guderley et al., 2006; Guderley et al., 2008) – it was used as the primary representative skeletal muscle.

Thus, the objectives of my study were:

1) To assess VO_{2max} in an HR_{mini} line (in which the "mini muscle" phenotype has become fixed), an HR_{normal} line (in which the "mini muscle" allele has been lost), and a non-selected control line;

2) To determine whole-body fuel use across a range of submaximal relative exercise intensities in HR_{mini}, HR_{normal}, and control mice;

3) To characterize some physical and metabolic cardiac properties in these mice; and

4) To evaluate several physiological factors involved in the regulation of skeletal muscle fuel use.

My ultimate aim was to determine if whole-body metabolic fuel use during exercise could be adaptively altered or differentially regulated if animals were under selection for high levels of voluntary locomotion. To this end, I tested the following hypotheses:

Hypothesis 1) HR_{mini} mice will have an enhanced whole-body ability to oxidize lipids, and will exhibit a shift in the fuel use pattern towards the utilization of a greater proportion of lipids than HR_{normal} or control mice at any given relative exercise intensity;

Hypothesis 2) If the fuel use pattern is conserved amongst all mice, the two phenotypes of HR mice (HR_{mini} and HR_{normal}) will diverge at some regulatory points in substrate uptake and metabolic pathways, thus achieving a common pattern of fuel use via differential mechanistic changes.

Materials and methods

Animals

This study used mice from an experimental evolution project, in which four closed lines are selectively bred for high voluntary locomotion (HR), and the other four lines contain non-selected controls. For details about the selective breeding regime and animal husbandry, refer to Swallow and colleagues (Swallow et al., 1998a). Animals in this study were from three of the eight lines: line 2 (a control line), line 3 (an HR line in which the "mini muscle" phenotype has become fixed) and line 8 (an HR line in which the "mini muscle" phenotype is not found). Mice from each line are referred to as control mice, HR_{mini} mice, and HR_{normal} mice, respectively.

For all cardiac measurements, hearts sampled from 8-9 week old male mice (n = 10) from generation 57 of selection were used. Mice from generation 58 were used for all *in vivo* and skeletal muscle measurements. These animals were 6-9 weeks old at the beginning of *in vivo* experiments. 30 mice (n = 10) were randomly distributed into two batches for exercise trials. 28 non-exercised mice (n = 10 for HR_{normal} and control mice, n = 8 for HR_{mini} mice), referred to as "pre-exercise" or sedentary mice, were also sampled with these two batches, so that age and other conditions were consistent.

During the course of the *in vivo* experiments, mice were housed individually at 22°C, and maintained on a 12:12 h light:dark cycle. Water and laboratory chow (8604 Teklad rodent diet, Harlan Laboratories) were available *ad libitum*. All experimental procedures were approved by the University of California Riverside Institutional Animal Care and Use Committee (AUP #A-20080018) and the McMaster University Animal Research Ethics Board.

In vivo: treadmill exercise and indirect calorimetry

In vivo experiments used to assess VO_{2max} and fuel use patterns across a range of submaximal exercise intensities were performed at the University of California Riverside (Riverside, CA), at room temperature (22-25°C). A positive-pressure flow-through respirometry system was used for all measurements of oxygen consumption rates (VO_2) and carbon dioxide consumption rates (VCO_2). Animals were force-exercised on a custom-made motorized treadmill (modified commercial unit from Columbus Instruments, Columbus, OH) at an incline of 10°, enclosed in a Plexiglass metabolic chamber (approximate volume 800 mL). Air entering the chamber was scrubbed of H_2O by Drierite (W.A. Hammond Drierite Company, Xenia, OH) and of CO_2 by soda lime and ascarite (Fisher Scientific, Pittsburgh, PA), and maintained at a constant flow of 2000 mL min^{-1} by a mass flow meter (Sable Systems International, Las Vegas, NV). Excurrent air was scrubbed of H_2O by magnesium perchlorate (Fisher Scientific), and subsampled at 200 mL min^{-1} by a CO_2 analyzer in series with an O_2 analyzer (FoxBox portable analysis instrument; Sable Systems International). Data were collected with a computer and data acquisition software (Expedata, Sable Systems International) at a sampling rate of 1 sample s^{-1}. The accuracy of this set-up was verified by burning methanol in the enclosed

chamber (McClelland et al., 1998), with the theoretical rates matching the actual rates within ± 1%.

Prior to the VO_{2max} trials, mice exercised for 10-15 min for two consecutive days, to become familiar with forced treadmill running. For familiarization trials, as with all subsequent VO_{2max} trials, mice had a 5 min adjustment period in the chamber before exercise began with a starting speed of 10 or 13 m min^{-1}, and incrementally increased by 3 m min^{-1} every 2 min. Aside from treadmill familiarization, there was a minimum of 24 h between exercise bouts for any individual mouse. The criteria for defining VO_{2max} included: 1) no change in VO_2 with increasing speed, 2) a respiratory exchange ratio (RER: VCO_2/VO_2) value ≥ 1.0, and 3) the mouse could no longer maintain position on the treadmill (McClelland et al., 1998; Seeherman et al., 1981). Trial quality was also assessed subjectively by assigning a score between 1 and 5 (with 5 being a perfect run), and "poor" runs (scores below 3) were repeated. For all mice, the maximal 10-15 s plateau of VO_2 was used for VO_{2max} analyses. VO_{2max} values are presented as mL O_2 h^{-1} per g body mass.

The order of the two submaximal exercise intensity trials (on average, 66% VO_{2max} and 78% VO_{2max}) was randomized for each mouse. Mice were fasted 6-8 h, to ensure a postabsorptive state during exercise. These trials were performed between 13.00 and 19.00 h. Each trial started with a 5 min adjustment period, and lasted for 25-30 min. For exercise of a higher absolute intensity (*i.e.* > 10 m min^{-1}), treadmill speed was increased from 10 m min^{-1} by incremental steps of 3 m min^{-1} every 1-2 min, until the designated speed was reached. For all submaximal exercise bouts, the appropriate speed was determined in real time, and was defined as the speed at which VO_2 relative to the individual's VO_{2max} (*i.e.* real-time relative exercise intensity) was approximately matched with the target relative exercise intensity (*i.e.* 65% VO_{2max} or 80% VO_{2max}); minor adjustments to treadmill speed were made during the exercise trial to maintain a constant relative exercise intensity. 60 s of continuous data from between minute 9-11 of the trial was used for analyses, to ensure that there were no temporal artifacts on RER.

Rates of VCO_2 and VO_2 (mL min^{-1}) were calculated using equations 1 and 2, respectively (equation 3b from Withers, 1977):

$$\mathbf{VCO_2} = V \bullet FECO_2 \quad (1)$$

$$\mathbf{VO_2} = [V \bullet (FIO_2 - FEO_2) - (VCO_2 \bullet FIO_2)] / (1 - FIO_2) \quad (2)$$

where $FECO_2$ is the fractional concentration of CO_2 in excurrent gas; FIO_2 and FEO_2 are the fractional O_2 concentrations in incurrent and excurrent gases, respectively; and V is the rate of airflow (mL min^{-1}) into the metabolic chamber.

Lipid and carbohydrate oxidation rates (g min^{-1}) were determined using indirect calorimetric equations 3 and 4 (Frayn, 1983), based on the assumption that protein contributions to total oxidation were negligible during aerobic exercise in the postabsorptive state (Rennie et al., 1981):

$$\textbf{Lipid oxidation rate} = (1.67 \bullet VO_2) - (1.67 \bullet VCO_2) \quad (3)$$

$$\textbf{Carbohydrate oxidation rate} = (4.55 \bullet VCO_2) - (3.21 \bullet VO_2) \quad (4)$$

with VO_2 and VCO_2 in L min^{-1}. Absolute fuel oxidation rates are presented as μmol O_2 h^{-1} per g body mass, based on the conversion factor of 22.4 L mol^{-1} for gases at standard

temperature and pressure. The proportional fuel contribution to total oxidation was designated as the absolute fuel oxidation rate relative to the total rate of O_2 consumption.

Tissue sampling

All tissue sampling took place between 13.00 and 18.00 h. Mice were fasted for 6-8 h and were euthanized by cervical dislocation, either within ~30 s of completing a 30 min exercise bout (at an average of 74% VO_{2max}), or without prior treadmill exercise ("pre-exercise" or sedentary mice). Within ~10 min of euthanization, all tissues (right and left gastrocnemius, plantaris, soleus, and tibialis anterior muscles) had been removed, weighed, and flash-frozen in dry ice with aluminum clamps. Tissues were stored at -80°C. Right and left ventricles were sampled in a similar manner, except that generation 57 mice were not fasted.

Samples were transported to McMaster University (Hamilton, ON) in a vapor shipper (Taylor-Wharton CX100 dry shipper). All tissues were powdered in liquid nitrogen with a mortar and pestle, and stored at -80°C until used for the following analyses. For these assays, chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise indicated.

Glycogen assay

Glycogen content was determined in the right side hindlimb muscles (gastrocnemius, soleus, plantaris, and tibialis anterior) of both pre- and post-exercise mice, using perchloric acid hydrolysis, amyloglucosidase digestion, and spectrophotometer quantification based on a hexokinase/glucose-6-phosphate dehydrogenase assay (Passonneau and Lauderdale, 1974; Suzuki et al., 2001).

Powdered tissue (whole muscle for soleus and plantaris, 20-40 mg of mixed tissue for gastrocnemius and tibialis anterior) was homogenized in 200 μL perchloric acid (6%) using a motorized homogenizer (PowerGen 125, Fisher Scientific). 100 μL of the homogenate was mixed with 50 μL $KHCO_3$ (1 M) and 100 μL acetate buffer (0.4 M, pH 4.8). Half of the mixture was set aside to measure pre-digestion glucose content, while 7 μL amyloglucosidase enzyme (4 U/μL) was added to the remaining 125 μL (and to 100 μL glycogen standards, 0 – 4.0 mM) for digestion. All samples, along with glycogen and glucose (0 – 2.0 mM) standards, were incubated in a shaking water bath at 40°C for 2 h, followed by neutralization with 1 M $KHCO_3$.

To quantify samples, 10 μL of each standard or sample (in triplicate) was mixed with 10 μL glucose-6-phosphate dehydrogenase (0.05 U/μL in TRA buffer: 0.3 M triethanolamine-HCl, 4.05 mM $MgSO_4$, pH 7.5; Roche Diagnostics, Indianapolis, IN) and 200 μL glucose assay solution (0.8 mM NADP, 10 mM ATP) in a 96-well plate. Absorbance was pre-read at 340 nm using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), and the plate was then incubated at 37°C for 10 min, prior to the addition of hexokinase (0.1 U/μL in TRA buffer; Roche Diagnostics). After a second incubation at 37°C for 30 min, the final absorbance was measured at 340 nm. Glycogen content (μmol) is presented both per g wet weight and per whole muscle.

Enzyme activity assays

Maximal activities of β-hydroxyacyl-CoA dehydrogenase (HOAD), hexokinase (HK), carnitine palmitoyltransferase II (CPT II), and citrate synthase (CS) were measured in left ventricles and in right gastrocnemius muscles, following previously described procedures (Houle-Leroy et al., 2000; Leonard, 1999; McClelland et al., 2004) with slight modifications. 20-50 mg of powdered tissue was diluted 1:20 (mg tissue:μL) with cold extraction buffer (100 mM K_2HPO_4/KH_2PO_4, 5 mM EDTA, 0.1% Triton X-100, pH 7.6) and homogenized for 1 min, using a cooled glass on glass homogenizer.

HOAD activity was measured in fresh homogenate that was further diluted with extraction buffer to a final concentration of 1:80 (mg:μL) in the case of cardiac tissue. Maximal HOAD activity was assayed at 340 nm in triethanolamine-HCl (TEA-HCl; 100 mM, pH 7.0), in the presence of NADH (0.28 mM) and ethylenediaminetetraacetic acid (EDTA; 5 mM), with acetoacetyl-CoA (0.1 mM) added as substrate. Maximal HK activity was also measured in fresh homogenate at 340 nm. This assay was performed using Hepes as the buffer (50 mM, pH 7.6), in the presence of $MgCl_2$ (8 mM), NADP (0.5 mM), ATP (8 mM), and excess levels of glucose-6-phosphate dehydrogenase (4 U), with glucose (5 mM) added as substrate.

For CS and CPT II, activity was measured after tissue homogenates were frozen and re-thawed. In the case of CS, tissue homogenates were further diluted with extraction buffer, to a final concentration of 1:100 (mg:μL) for gastrocnemius or 1:240 (mg:μL) for ventricles. Maximal CS activity, measured at 412 nm, was assayed in Tris-HCl (100 mM, pH 8.0), in the presence of 2,2'-nitro-5,5'-dithiobenzoic acid (DTNB; 0.1 mM) and acetyl-CoA (0.3 mM), with oxaloacetate (0.25 mM) added as substrate. Maximal CPT II activity, measured at 412 nm, was assayed in Tris-HCl (40 mM, pH 8.0), in the presence of DTNB (0.2 mM), EDTA (1.5 mM) and palmitoyl-CoA (0.05 mM), with L-carnitine (5 mM) added as substrate. This assay measures CPT II activity because CPT I is inactivated by freezing and detergent treatment (see McClelland et al., 2004).

All maximal enzyme activity levels were assayed at 37°C in 96-well plates using a SpectraMax Plus 384 spectrophotometer (Molecular Devices). Assays were performed in triplicate, with an additional negative control well (lacking substrate) to correct for background activity. Enzyme activity data are presented as the mean rate of conversion of substrate to product (Unit = $\mu mol\ min^{-1}$).

DNA quantification

Total DNA content was quantified in the left ventricles (as previously described in Templeman et al., 2010). It was measured in tissue homogenates that had been used for the enzyme activity assays, after they were frozen and re-thawed, and further diluted to 1:40 (mg tissue:μL extraction buffer). In brief, aliquots of tissue homogenates in 2x digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, 0.2 mg mL^{-1} proteinase K, pH 8.0) were digested overnight (18 h) at 55°C. A DNA standard curve (1 – 100 ng μL^{-1}) was constructed from the purified (via phenol extraction and ethanol precipitation) and re-suspended DNA from a mixture of these digested samples. Duplicates of each DNA standard re-suspended in TE buffer (10 mM Tris-HCl, 1 mM

EDTA, pH 7.5) and triplicates of digested samples were loaded onto a 96-well black fluorometric plate with PicoGreen (diluted 1:400 in TE buffer). The plate was incubated for five minutes at room temperature, in the dark. Fluorescence was then measured (excitation 480 nm, emission 535 nm, cut-off 530 nm) by a SpectraMax Gemini XPS fluorescence spectrophotometer (Molecular Devices). DNA content (mg) is presented per g wet weight and per whole ventricle.

Sample preparation for ELISA

The cytosolic fraction of left gastrocnemius tissue was prepared as previously described (Butz et al., 2004; Dubouchaud et al., 2000; McClelland and Brooks, 2002), with slight modifications. 50-100 mg of powdered tissue was homogenized for 1 min with a cooled glass on glass homogenizer, in a buffer containing Hepes (30 mM), sucrose (210 mM), ethylene glycol tetraacetic acid (EGTA; 2 mM), NaCl (40 mM), and a protease inhibitor cocktail (Complete MINI, Roche Diagnostics), at pH 7.4. This homogenate was centrifuged at 600 *g* for 10 min (4°C) to remove erythrocyte material, and then supernatant from this step was centrifuged at 10,000 *g* for 20 min (4°C). The ensuing supernatant (with the mitochondrial fraction removed) was diluted (0.75 x volume) with a buffer containing KCl (1.167 M) and Na_4PPi (58.3 mM), at pH 7.4, and centrifuged at 230,000 *g* for 2 h (4°C). The resulting supernatant was collected as pure cytosol, and aliquots were frozen at -80°C.

H-FABP ELISA

Heart-type fatty acid binding protein (H-FABP) content was determined in cytosolic fractions of left ventricles, using an enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle (see Vork et al., 1991). Total protein content in the cytosol samples was quantified with the commercial Pierce BCA Protein Assay kit (Thermo Scientific, Whitby, ON, Canada). H-FABP content was measured in equal amounts of total cytosolic protein (0.015 μg) for each sample, using a commercial ELISA kit designed for mouse H-FABP (HK403, Hycult Biotech). H-FABP protein levels are expressed relative to total cytosolic protein content.

Real-time PCR

In the left and right ventricles, real-time PCR was used for the relative quantification of peroxisome proliferator-activated receptor (*PPAR*) α, medium-chain acetyl-CoA dehydrogenase (*MCAD*), and muscle- and liver-type *CPT-1* (*Cpt-1β* and *Cpt-1α*, respectively) mRNA levels was performed in right and left ventricles, using TATA-binding protein (*TBP*) as the housekeeping gene (Templeman et al., 2010). Relative quantification of the metabolic transcriptional factors *PPARα*, peroxisome proliferator-activated receptor gamma coactivator 1-α (*PGC-1α*), *PPARβ/δ*, NAD-dependent deacetylase sirtuin-1 (*SIRT1*), and also of fatty acid translocase (*FAT/CD36*) was performed in the left gastrocnemius muscles, using *18S* ribosomal protein as the housekeeping gene. *18S* was selected as an internal control for these samples because it

is a relatively stable housekeeping gene and skeletal muscle *18S* rRNA levels are not affected by endurance exercise (Siu et al., 2004; Thellin et al., 1999). Primer sequences (Table 2.1) were either previously published sequences (Asher et al., 2008; Templeman et al., 2010) or designed using Primer 3 software. The specificity of primer pairs (Mobix, Hamilton, ON, Canada) was tested separately for each mouse line, using PCR and gel electrophoresis.

RNA from 15-40 mg of tissue was extracted by homogenization with TRIzol reagent (Invitrogen, Burlington, ON), based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentration of each sample was measured in duplicate, using a NanoDrop ND-1000 spectrophotometer (Fisher Scientific) to quantify RNA at 260 nm by UV spectrophotometry. RNA purity was verified by assessing the ratio of absorbances at 260:280 nm, with a minimum value of 1.90. To generate cDNA, 1 μg of total RNA was treated with DNase I, and then reverse transcription to cDNA was carried out by SuperScript II RNase H^- reverse transcriptase with random primers and Oligo dT primers, in the presence of a dNTP mixture (Invitrogen).

5 μL of 1:5 (cDNA:μL water) diluted cDNA was mixed with 12.5 μL SYBR green (Bio-Rad Laboratories, Mississauga, ON), 5.5 μL RNase/DNase-free water, and 1 μL each of forward and reverse primers (5 μM) for quantitative real-time PCR reactions. A negative control (containing water in lieu of cDNA) was run on each plate to ensure a lack of contamination. Reactions were performed in duplicate on a Stratagene MX3000P QPCR system (Stratagene, La Jolla, CA, USA), which detected SYBR green with ROX as the reference dye. In all cases, the thermal program consisted of a 3 min initial denaturation at 95°C, then 40 cycles of 95°C for 15 s, 60°C for 45 s, and 72°C for 30 s. A dissociation curve analysis was performed to verify the specificity of PCR products. For each target gene, the amplification efficiency (E) was determined using the slope of a standard curve (see Cikos et al., 2007) constructed from serial dilutions of pooled cDNA, using an equal mix of all mouse phenotypes:

$$E = 10^{-1/\text{Slope}} - 1 \quad (5)$$

The relative mRNA levels of each sample was then calculated based on the amplification efficiency, using the comparative Ct method (Cikos et al., 2007):

$$\text{mRNA quantity} = 1 / (E + 1)^{\text{Ct}} \quad (6)$$

where Ct is the number of amplification cycles to reach the fluorescence threshold. All values were normalized against mRNA levels of the housekeeping gene, which did not differ between groups ($p > 0.2$ for each housekeeping gene), and expressed relative to mean control values (arbitrarily set at 1).

Statistics

All statistical analyses were performed using SPSS 11.0 software. Analysis of covariance (ANCOVA) models were used for each dependent variable, to determine the effect of the fixed factor "mouse phenotype" (HR_{mini}, HR_{normal}, control). Because body mass differed between lines, body mass was always included as a covariate, except when it had been accounted for in the dependent variable (*e.g.* VO_{2max} per g body mass), or when the analysis was meant to determine the effect of mouse phenotype on absolute

ventricle or heart sizes. However, it is not clear whether statistical analyses should adjust for body size when testing metabolic variables, such as enzyme activity levels (Houle-Leroy et al., 2000). Therefore, analyses of glycogen content, maximal enzyme activity, H-FABP, and mRNA content were also performed without body mass as a covariate (Houle-Leroy et al., 2000; Houle-Leroy et al., 2003), and this did not notably alter results. In all analyses, age, batch, and dam identity (as a limited number of the mice were siblings) were also tested as covariates, and were removed from the ANCOVA model if they had no significant effect.

Absolute substrate oxidation rates and the proportional contributions of fuels to total oxidation were analyzed at two separate exercise intensities, designated as 66% VO_{2max} and 78% VO_{2max}. However, to account for individual variation in the relative exercise intensity of each exercise bout, the covariate of "relative exercise intensity" (*i.e.* VO_2 of the run relative to individual VO_{2max}) was incorporated into these ANCOVA models.

To determine if there was significant exercise-induced glycogen depletion in hindlimb muscles, an additional fixed factor of "exercise" was added to the model, to test the effect of pre- versus post-exercise glycogen content in a 2-way ANCOVA. When there was a significant interaction between the main effects of "mouse phenotype" and "exercise," the data were split by mouse phenotype, and separate 1-way ANCOVAs were performed within each mouse phenotype to determine how exercise affected glycogen content. In these cases, the main effect of mouse phenotype could not be interpreted.

For analyses in which a significant effect was detected, either pairwise comparisons with a Bonferroni correction (for ANCOVA models) or Tukey's post hoc testing (for ANOVA models, when all covariates were removed) was used to compare mouse phenotypes. For all statistical analyses, the critical α-level was set at $p = 0.05$.

Table 2.1: Primer sequences used for real-time PCR mRNA quantification in mouse ventricles and/or gastrocnemius.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Cpt-1α[1]	AAACCCACCAGGCTACAGTG	TCCTTGTAATGTGCGAGCTG
Cpt-1β[1]	CCCATGTGCTCCTACCAGAT	CCTTGAAGAAGCGACCTTTG
FAT/CD36	CAACTGGTGGATGGTTTCCT	GCAGAATCAAGGGAGAGCAC
MCAD[1]	TCGGAGGCTATGGATTCAAC	TCAATGTGCTCACGAGCTATG
PGC-1α	ATGTGTCGCCTTCTTGCTCT	ATCTACTGCCTGGGGACCTT
PPARα[1]	TCATACATGACATGGAGACCTTG	ACTGGCAGCAGTGGAAGAATC
PPARβ/δ	GGTAGAAGCCATCCAGGACA	CCGTCTTCTTTAGCCACTGC
SIRT1[2]	GATACCTTGGAGCAGGTTGC	CTCCACGAACAGCTTCACAA
TBP[1]	GGCCTCTCAGAAGCATCACTA	GCCAAGCCCTGAGCATAA
18S	TGTGGTGTTGAGGAAAGCAG	TCCCATCCTTCACATCCTTC

[1] (Templeman et al., 2010)
[2] (Asher et al., 2008)

Results

In vivo: VO_{2max} and fuel use during exercise

Both lines of HR mice exhibited a greater mass-specific maximal aerobic capacity (VO_{2max}, expressed per g body mass) than non-selected control mice, with the VO_{2max} of HR_{mini} mice slightly enhanced above that of HR_{normal} mice ($p < 0.05$; Fig. 2.1). This variation in aerobic capacity was accounted for in subsequent exercise bouts, as mice were exercised at common relative intensities (*i.e.* relative to each individual's mass-specific VO_{2max}).

During low intensity exercise (for all mice, an average of 66% VO_{2max}; 66 ± 1% VO_{2max} for controls, 64 ± 2% VO_{2max} for HR_{mini} mice, and 68 ± 2% VO_{2max} for HR_{normal} mice), approximately 70% of energy was supplied via the oxidation of lipids, with the remaining 30% fueled by carbohydrates (Fig. 2.3A). The increased total energy consumption of HR mice exercising at 66% VO_{2max} (necessitated by their higher aerobic capacities) appears to have been largely supplied by an increased flux of lipids, with absolute lipid oxidation rates of 215 ± 11 μmol O_2 hr^{-1} g^{-1} for HR_{mini} mice and 229 ± 16 μmol O_2 hr^{-1} g^{-1} for HR_{normal} mice, compared to 183 ± 13 μmol O_2 hr^{-1} g^{-1} for control mice ($p < 0.05$; Fig. 2.2B). The rates of carbohydrate oxidation were not significantly different between mouse phenotypes (90 ± 10 μmol O_2 hr^{-1} g^{-1} for control mice, 91 ± 8 μmol O_2 hr^{-1} g^{-1} for HR_{mini} mice, and 82 ± 9 μmol O_2 hr^{-1} g^{-1} for HR_{normal} mice; Fig. 2.2A). However, when fuel oxidation rates were expressed relative to total VO_2 during exercise, there were no statistically significant differences in the mix of fuels supplying oxidation at 66% VO_{2max} (Fig. 2.3A).

During moderately high intensity exercise (for all mice, an average of 78% VO_{2max}; 78 ± 1% VO_{2max} for controls, 76 ± 1% VO_{2max} for HR_{mini} mice, and 79 ± 1% VO_{2max} for HR_{normal} mice), the absolute lipid oxidation rates in control mice were equivalent to those in HR mice (196 ± 13 μmol O_2 hr^{-1} g^{-1} for control mice, 197 ± 14 μmol O_2 hr^{-1} g^{-1} for HR_{mini} mice, and 197 ± 18 μmol O_2 hr^{-1} g^{-1} for HR_{normal} mice; Fig. 2.2D). At this increased intensity, carbohydrate flux in all mice reached a level where carbohydrates contributed to more than 40% of total oxidation, at 126 ± 13 μmol O_2 hr^{-1} g^{-1} for control mice, 165 ± 12 μmol O_2 hr^{-1} g^{-1} for HR_{mini} mice, and 163 ± 19 μmol O_2 hr^{-1} g^{-1} for HR_{normal} mice (Figs. 2.2C and 2.3B). There is a trend suggesting elevated absolute carbohydrate oxidation rates in HR mice at 78% VO_{2max}, but it is not statistically significant ($p = 0.098$; Fig. 2.2C).

Cardiac properties

The characterization of some of physical, molecular, and biochemical properties of control and HR mouse hearts revealed several physiological differences. Relative to body mass, HR_{mini} mice had significantly larger left ventricles (and thus whole hearts) than both control and HR_{normal} mice ($p \leq 0.001$; Table 2.2). This seemed to primarily result from HR_{mini} mice having a reduced body mass ($p < 0.05$; Table 2.4), although the

slight enlargement of whole heart size in HR_{mini} mice approached significance even without the inclusion of body mass as a covariate ($p = 0.096$; Table 2.2). There were no significant differences in left ventricle DNA content, whether on a per g wet weight or per whole ventricle basis, which further supports a lack of significant cardiac hypertrophy (Table 2.2). In addition to these physical changes, both lines of HR mice exhibited some degree of cardiac metabolic remodeling compared to control mice, with increased maximal HK activity ($p < 0.05$; Fig. 2.4), concurrent to a reduction in the maximal activity of CPT II in HR_{mini} mice ($p < 0.05$) that approached significance in HR_{normal} mice ($p = 0.100$; Fig. 2.4). However, there were no significant differences in the mRNA content of *PPARα*, *MCAD*, *CPT-1α*, or *CPT-1β* in either the right or left ventricles of HR_{mini}, HR_{normal}, or control mice (Table 2.3).

Hindlimb muscle masses

Several of the hindlimb muscles of HR_{mini} mice were significantly reduced in size compared to the "normal muscle" phenotype, specifically the gastrocnemius, the plantaris, and the tibialis anterior ($p < 0.05$; Table 2.4). These reduced muscles masses likely contribute to the reduced whole body mass of HR_{mini} mice, compared to HR_{normal} and control mice ($p < 0.05$; Table 2.4). On the other hand, selectively breed HR mice referred to as "HR_{normal}" mice (due to their lack of this "mini muscle" phenotype) also showed some muscle size divergence from control mice, in particular with respect to the slightly (but significantly) larger size of the gastrocnemius ($p < 0.05$; Table 2.4).

Muscle glycogen content

In the gastrocnemius and plantaris, HR_{mini} had significantly greater mass-specific glycogen stores than HR_{normal} or control mice, with as much as twice the amount of glycogen stored per g wet weight in some of these hindlimb muscles ($p < 0.05$; Fig. 2.5). It is possible that this was also the case in the tibialis anterior, but a significant statistical interaction precluded the interpretation of the main effect of mouse phenotype. For all muscles, when accounting for the large variation in hindlimb muscle masses between mouse phenotypes (see Table 2.4), it was revealed that there were no significant differences in total glycogen stores on a whole muscle basis (Fig. 2.6).

There were varying patterns of muscle glycogen depletion between the mouse phenotypes after a common exercise regime (30 min, an average of 74% VO_{2max} for all mice; 74 ± 1% VO_{2max} for controls, 73 ± 1% VO_{2max} for HR_{mini} mice, and 72 ± 2% VO_{2max} for HR_{normal} mice). Significant differences between pre- and post-exercised muscle glycogen content were generally consistent between per g wet weight and per whole muscle measurements. For instance, HR_{normal} and control mice, but not HR_{mini} mice, exhibited exercise-induced glycogen depletion in the tibialis anterior (per g w.w.: $p < 0.05$ for HR_{normal} mice, $p = 0.057$ for controls; per whole muscle: $p < 0.05$ for both mouse phenotypes; Figs. 2.5B and 2.6B). While all mouse phenotypes had exercise-induced glycogen depletion in the plantaris ($p \leq 0.001$; Figs. 2.5C and 2.6C), only HR_{mini} and control mice had significantly reduced glycogen in the soleus of post-exercise animals (per g w.w.: $p < 0.05$ for both mouse phenotypes; per whole muscle: $p < 0.05$ for

controls, $p = 0.101$ for HR_{mini} mice; Figs. 2.5D and 2.6D). Unlike other hindlimb muscles, there was no significant glycogen depletion in the gastrocnemius due to exercise, despite a trend suggesting glycogen depletion in the whole muscle of HR_{normal} mice (Figs. 2.5A and 2.6A).

Facilitators of fatty acid uptake: H-FABP protein and *FAT/CD36* mRNA

Resting HR_{mini} mice had nearly double the amount of cytosolic H-FABP protein (per mg total cytosolic protein) of HR_{normal} or control mice in the gastrocnemius ($p < 0.05$; Fig. 2.7A). Although sarcolemmal protein content of FAT/CD36 in these mice has not been successfully measured to date, *FAT/CD36* mRNA levels were also greatly augmented in the gastrocnemius of resting HR_{mini} mice, at nearly three-fold higher than the gene transcript content in HR_{normal} or control mice ($p \leq 0.001$; Fig. 2.7B).

Metabolic enzyme activities

Both HR_{mini} and HR_{normal} mice appeared to diverge from control mice with respect to the maximal activities of several metabolic enzymes in the gastrocnemius of resting animals. The mass-specific maximal activities of CS, HOAD, and HK were greatly augmented in the gastrocnemius of HR_{mini} mice, compared to mice with a normal muscle phenotype ($p < 0.01$; Fig. 2.8A). In fact, due to mass-specific enzyme activities nearly double those of control mice (Fig. 2.8A), in a gastrocnemius less than half the mass (Table 2.4), HR_{mini} mice had equivalent total maximal enzyme activities as control mice, in the whole muscle (Fig. 2.8B). Conversely, while HR_{normal} mice did not have notably altered mass-specific enzyme activities compared to control mice, on a whole muscle basis they exhibited significantly greater maximal activities of CS, HOAD, CPT II, and HK compared to HR_{mini} (with the exception of HOAD, $p = 0.140$) and control mice ($p < 0.05$; Fig. 2.8B). This elevation in the total metabolic enzyme activity of the gastrocnemius was predominately reflective of the increased mass of that muscle in HR_{normal} mice (Table 2.4).

Gene expression and transcriptional regulation

Lastly, I examined gene expression of several transcriptional factors that are instrumental in mediating metabolism. While mRNA levels of PGC-1α, PPARβ/δ, and SIRT1 were not significantly different between mouse phenotypes, PPARα mRNA content was approximately 2.5-fold greater in the resting gastrocnemius of HRmini mice than in HRnormal or control mice ($p < 0.05$; Fig. 2.9A.). Furthermore, transcript levels of FAT/CD36 were closely correlated with PPARα amongst all mice ($r^2 = 0.84$, $p \leq 0.001$; Fig. 2.9B).

Figure 2.1: Maximal aerobic capacity of HR_{mini}, HR_{normal}, and control mice.

Maximal aerobic capacity (VO_{2max} per g body mass) of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice (n = 10). Data are presented as means ± SEM. Bars not sharing a common letter are significantly different ($p < 0.05$).

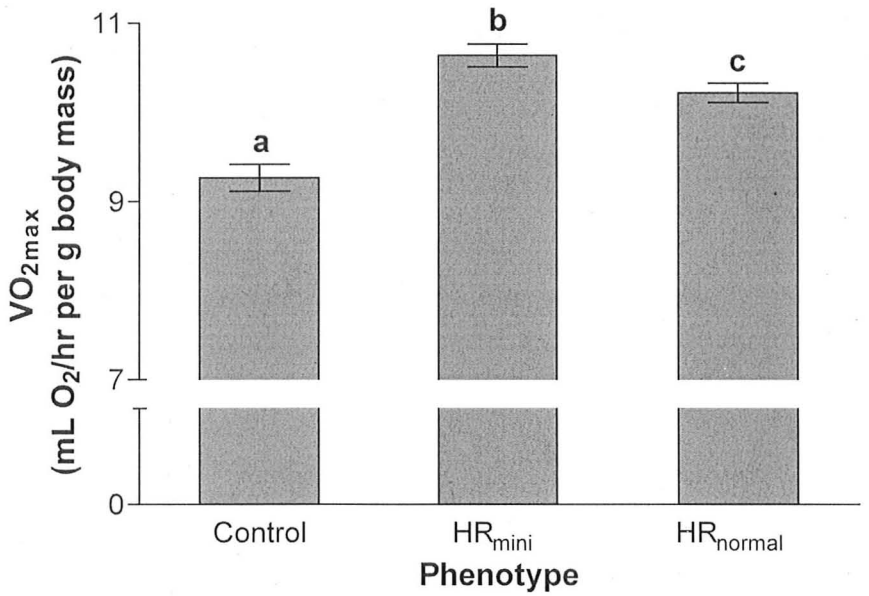

11
9
7
0
a
b
c
VO_{2max}
(mL O_2/hr per g body mass)
Control
HR_{mini}
HR_{normal}
Phenotype

Figure 2.2: Absolute oxidation rates of carbohydrates and lipids during exercise in HR_{mini}, HR_{normal}, and control mice.

Whole-body oxidation rate (relative to body mass) of (A. and C.) carbohydrates and (B. and D.) lipids, at an average exercise intensity of (A. and B.) 66% VO_{2max} and (C. and D.) 78% VO_{2max}, in non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice (n = 9-10). Data are presented as means ± SEM. For each variable, the presence of letters denotes statistical significance, where bars not sharing a common letter are significantly different ($p < 0.05$).

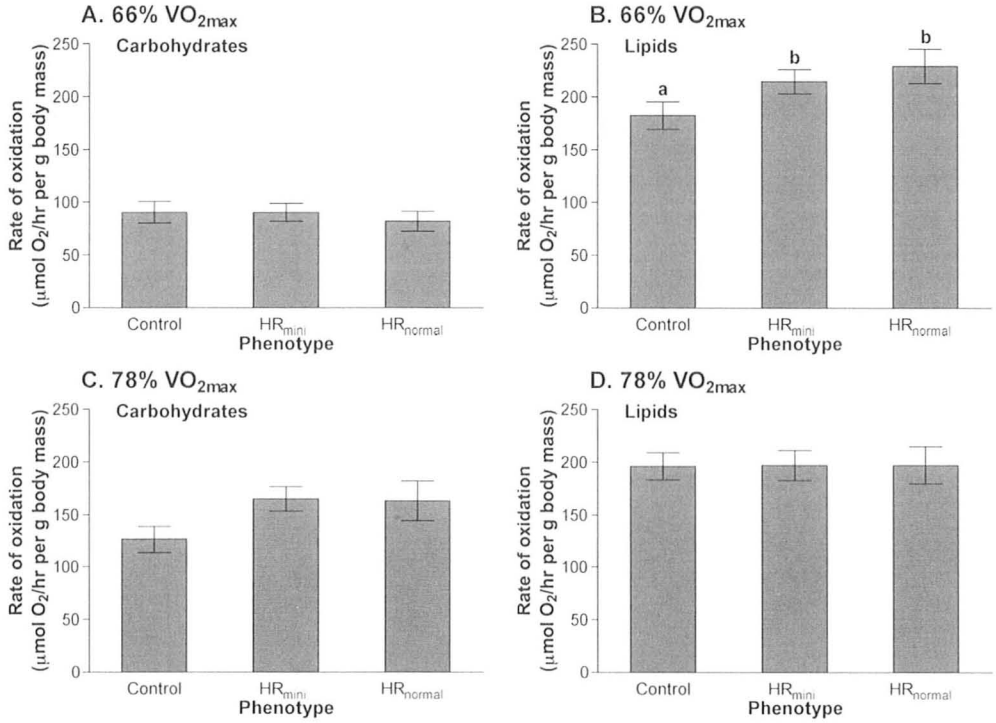

A. 66% VO2max
Carbohydrates
B. 66% VO2max
Lipids
C. 78% VO2max
Carbohydrates
D. 78% VO2max
Lipids
Rate of oxidation (μmol O2/hr per g body mass)
250
200
150
100
50
0
a
b
b
Control
HRmini
HRnormal
Phenotype

Figure 2.3: Proportional contribution of carbohydrates and lipids to total oxidation during exercise in HR_{mini}, HR_{normal}, and control mice.

Proportionate contributions to whole-body oxidation rates by carbohydrates and lipids at average exercise intensities of (A.) 66% VO_{2max} and (B.) 78% VO_{2max}, in non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice (n = 9-10). Data are presented as means ± SEM.

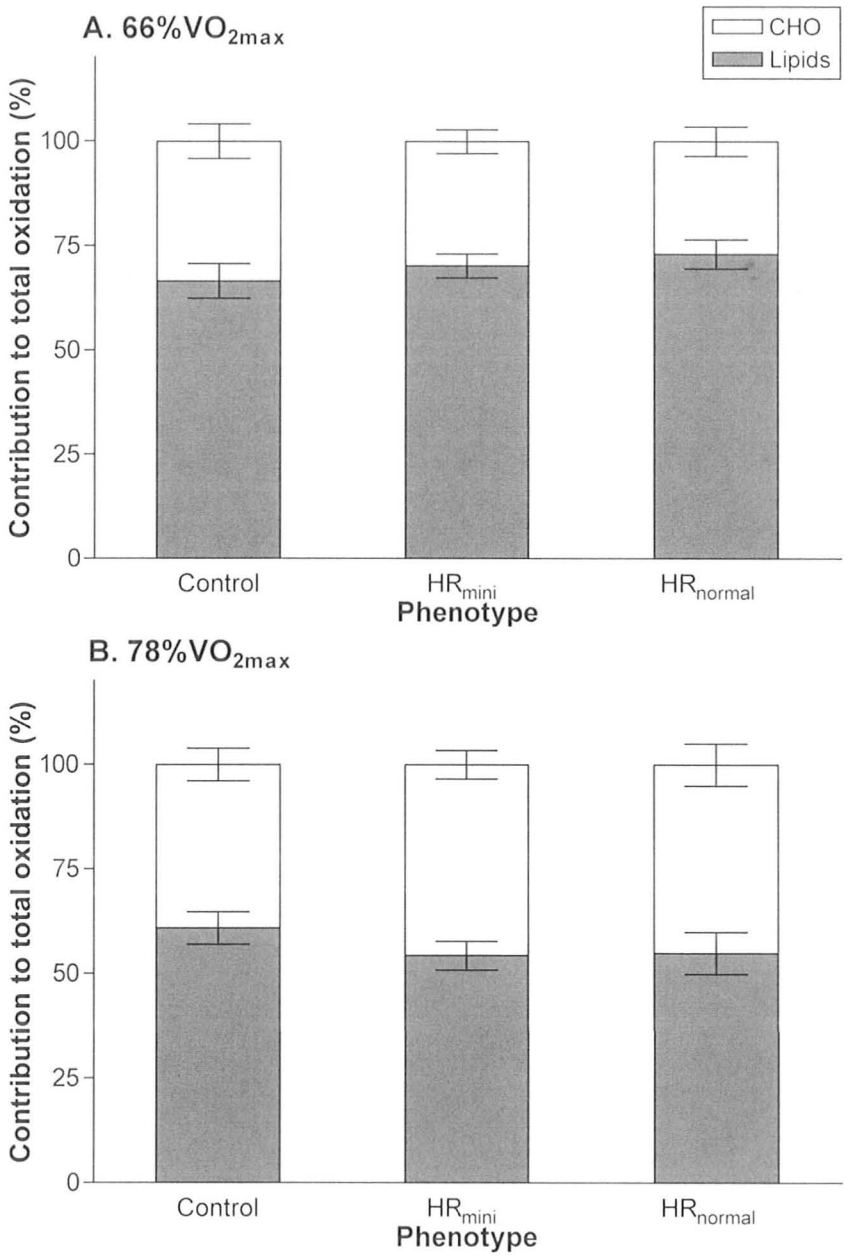

A. 66%VO2max
CHO
Lipids
Contribution to total oxidation (%)
100
75
50
25
0
Control
HRmini
HRnormal
Phenotype
B. 78%VO2max
Contribution to total oxidation (%)
100
75
50
25
0
Control
HRmini
HRnormal
Phenotype

Table 2.2: Physical and molecular cardiac characteristics of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice.

	Control	HR_{mini}	HR_{normal}
Relative left ventricle mass (mg/g body mass)	3.24 ± 0.07 ^{a} (10)	3.68 ± 0.06 ^{b} (10)	3.29 ± 0.06 ^{a} (10)
Relative right ventricle mass (mg/g body mass)	0.63 ± 0.02 (10)	0.75 ± 0.04 (10)	0.70 ± 0.06 (10)
Relative whole heart mass (mg/g body mass)	3.87 ± 0.06 ^{a} (10)	4.43 ± 0.09 ^{b} (10)	3.99 ± 0.08 ^{a} (10)
Whole heart mass (mg)	124.4 ± 2.8 (10)	133.4 ± 2.7 (10)	126.8 ± 3.1 (10)
Left ventricle DNA (mg/g w.w.)	5.59 ± 0.42 (8)	5.65 ± 0.38 (8)	4.99 ± 0.43 (8)
Total left ventricle DNA (mg/left ventricle)	0.60 ± 0.05 (8)	0.62 ± 0.04 (8)	0.50 ± 0.06 (8)

Note: Data are presented as means ± SEM, with sample size in parenthesis;
For each variable, the presence of letters denotes statistical significance,
where values not sharing a common letter are significantly different ($p \leq 0.001$).

Figure 2.4: Metabolic enzyme activities in the left ventricles of HR_{mini}, HR_{normal}, and control mice.

Maximal enzyme activities (relative to tissue wet weight) for CS, HOAD, CPT II, and HK in the left ventricle of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice (n = 7-8). Data are presented as means ± SEM. For each enzyme, the presence of letters denotes statistical significance, where bars not sharing a common letter are significantly different ($p < 0.05$). U = $\mu mol\ min^{-1}$.

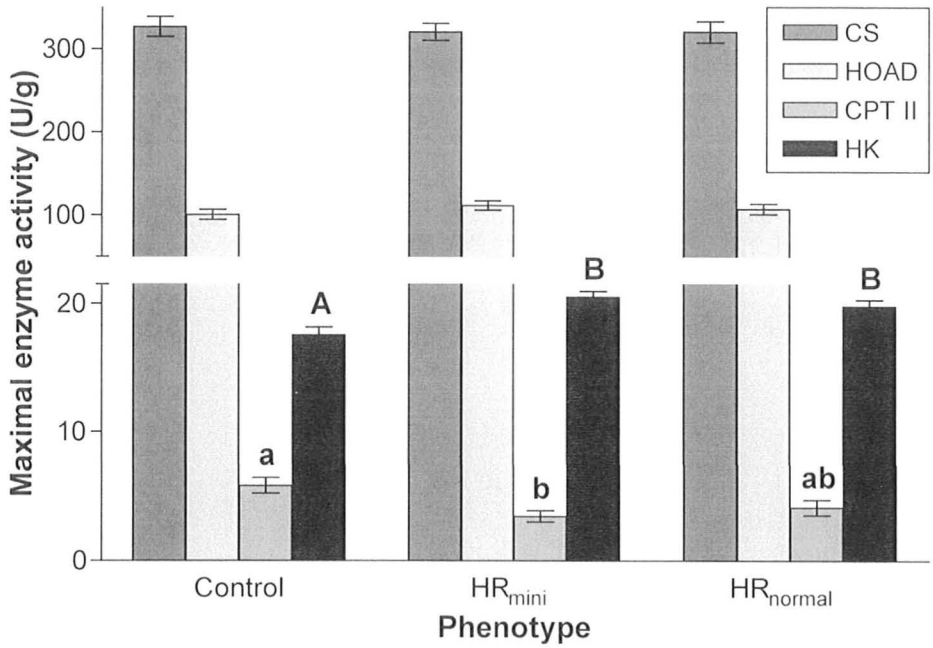

CS
HOAD
CPT II
HK
Maximal enzyme activity (U/g)
300
200
100
20
10
0
A
a
B
b
B
ab
Control
HRmini
HRnormal
Phenotype

Table 2.3: mRNA levels of metabolic genes in the hearts of HR_{mini}, HR_{normal}, and control mice.

Relative gene expression for *PPARα* and the PPARα-transcriptionally regulated enzymes *MCAD* and liver- and muscle-type *CPT-1* (*CPT-1α* and *CPT-1β*, respectively) in the left and right ventricles of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice.

		Control	HR_{mini}	HR_{normal}
***PPARα* relative mRNA**	**Left Ventricle**	1.00 ± 0.18 (7)	0.78 ± 0.03 (7)	1.05 ± 0.14 (8)
	Right Ventricle	1.00 ± 0.09 (6)	0.88 ± 0.10 (7)	1.28 ± 0.20 (8)
***MCAD* relative mRNA**	**Left Ventricle**	1.00 ± 0.13 (7)	0.99 ± 0.11 (7)	0.94 ± 0.13 (8)
	Right Ventricle	1.00 ± 0.14 (7)	0.94 ± 0.14 (8)	1.31 ± 0.23 (8)
***CPT-1α* relative mRNA**	**Left Ventricle**	1.00 ± 0.22 (7)	0.74 ± 0.10 (7)	0.79 ± 0.10 (8)
	Right Ventricle	1.00 ± 0.17 (6)	0.92 ± 0.18 (7)	1.42 ± 0.32 (8)
***CPT-1β* relative mRNA**	**Left Ventricle**	1.00 ± 0.17 (7)	0.86 ± 0.06 (7)	0.82 ± 0.06 (8)
	Right Ventricle	1.00 ± 0.09 (6)	0.88 ± 0.10 (7)	1.28 ± 0.20 (8)

Note: Data are presented as means ± SEM, with sample size in parenthesis; mRNA levels are expressed corrected against *TBP*, and normalized to mean control values (arbitrarily set at 1.00).

Table 2.4: Whole body and hindlimb muscle masses of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice.

	Control	HR_{mini}	HR_{normal}
Body mass (g)	29.8 ± 0.6 ^{a} (20)	27.5 ± 0.6 ^{b} (18)	30.1 ± 0.5 ^{a} (20)
Gastrocnemius mass (mg)	125.1 ± 4.0 ^{a} (20)	58.7 ± 2.2 ^{b} (17)	134.7 ± 2.8 ^{c} (20)
Plantaris mass (mg)	11.5 ± 0.8 ^{a} (19)	7.9 ± 0.6 ^{b} (16)	12.7 ± 0.5 ^{a} (20)
Soleus mass (mg)	7.3 ± 0.5 (19)	7.3 ± 0.7 (18)	6.5 ± 0.3 (10)
Tibialis anterior mass (mg)	52.5 ± 1.8 ^{a} (20)	42.4 ± 2.1 ^{b} (18)	52.3 ± 2.2 ^{a} (19)

Note: Data are presented as means ± SEM, with sample size in parenthesis;
For each variable, the presence of letters denotes statistical significance,
where values not sharing a common letter are significantly different ($p < 0.05$).

Figure 2.5: Mass-specific glycogen content in hindlimb muscles of pre- and post-exercise HR_{mini}, HR_{normal}, and control mice.

Glycogen content (relative to tissue wet weight) in the (A.) gastrocnemius, (B.) tibialis anterior, (C.) plantaris, and (D.) soleus of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice, either pre-exercise (n = 7-10) or post-exercise (30 min, average intensity 74% VO_{2max}; n = 9-10). Data are presented as means ± SEM. For each muscle, the presence of letters denotes statistical significance, where mouse phenotypes not sharing a common letter are significantly different; asterisks represent significant differences between pre- and post-exercise glycogen levels ($p < 0.05$).

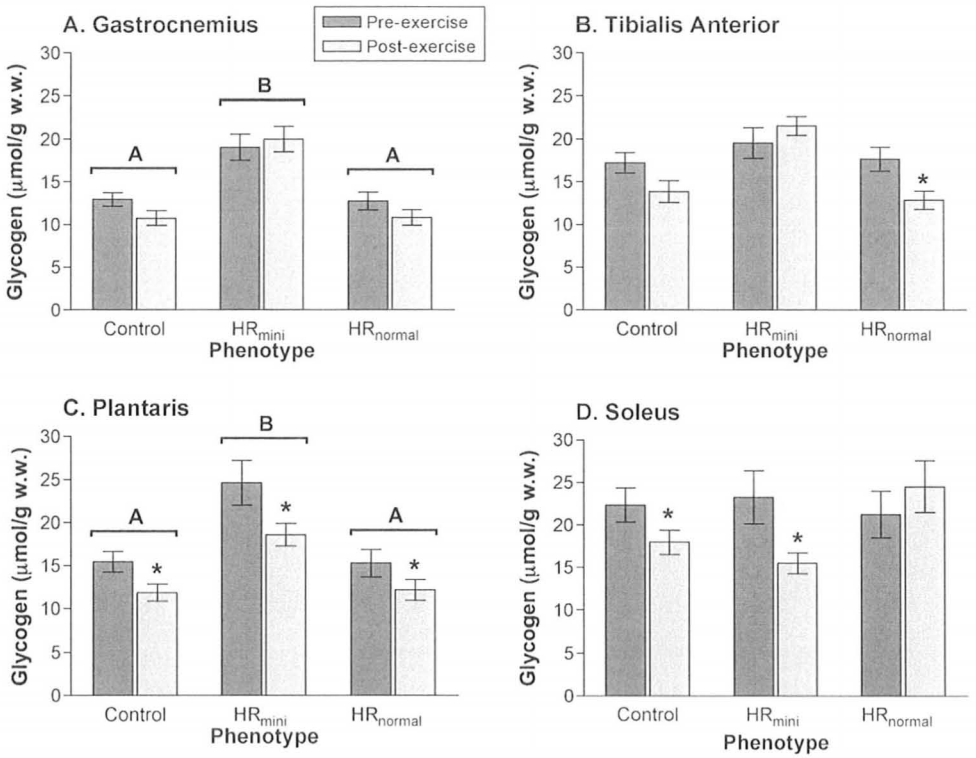

A. Gastrocnemius
Pre-exercise
Post-exercise
Glycogen (μmol/g w.w.)
30
25
20
15
10
5
0
A
B
A
Control
HRmini
HRnormal
Phenotype
B. Tibialis Anterior
Glycogen (μmol/g w.w.)
*
Control
HRmini
HRnormal
Phenotype
C. Plantaris
Glycogen (μmol/g w.w.)
A
B
A
*
*
*
Control
HRmini
HRnormal
Phenotype
D. Soleus
Glycogen (μmol/g w.w.)
*
*
Control
HRmini
HRnormal
Phenotype

Figure 2.6: Total glycogen content in hindlimb muscles of pre- and post-exercise HR_{mini}, HR_{normal}, and control mice.

Total glycogen content (per whole muscle) in the (A.) gastrocnemius, (B.) tibialis anterior, (C.) plantaris, and (D.) soleus of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice, either pre-exercise (n = 7-10) or post-exercise (30 min, average intensity 74% VO_{2max}; n = 9-10). Data are presented as means ± SEM. Asterisks represent significant differences between pre- and post-exercise glycogen levels ($p < 0.05$).

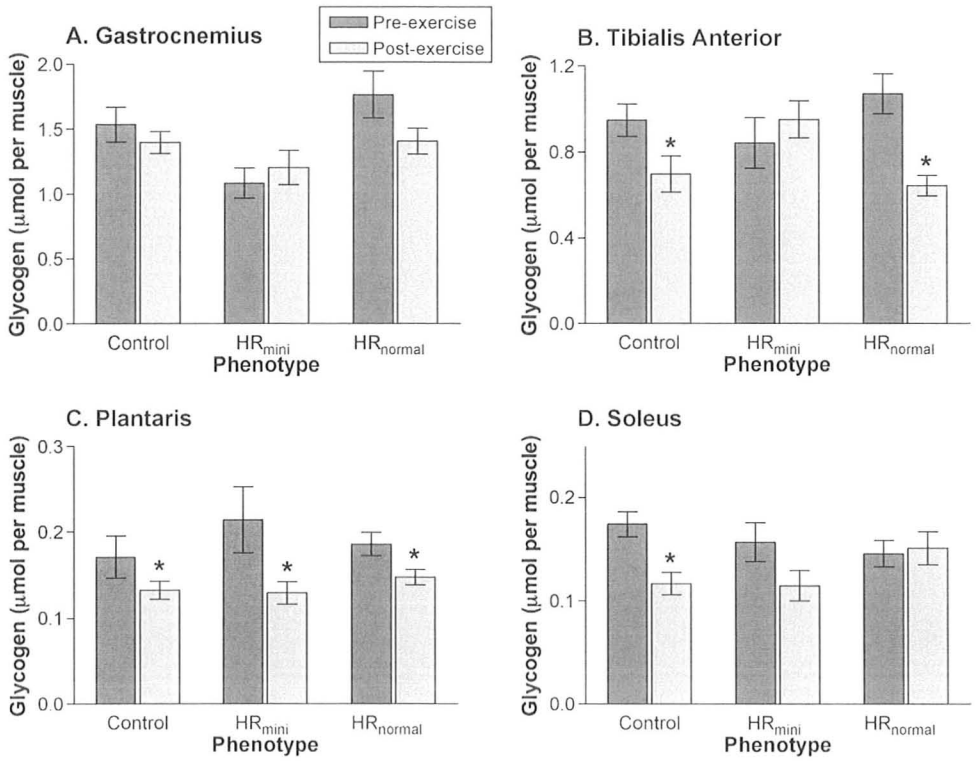

A. Gastrocnemius
Pre-exercise
Post-exercise
Glycogen (μmol per muscle)
2.0
1.5
1.0
0.5
0.0
Control
HRmini
HRnormal
Phenotype
B. Tibialis Anterior
Glycogen (μmol per muscle)
1.2
0.8
0.4
0.0
Control
HRmini
HRnormal
Phenotype
C. Plantaris
Glycogen (μmol per muscle)
0.3
0.2
0.1
0.0
Control
HRmini
HRnormal
Phenotype
D. Soleus
Glycogen (μmol per muscle)
0.2
0.1
0.0
Control
HRmini
HRnormal
Phenotype

Figure 2.7: Gastrocnemius H-FABP protein content and *FAT/CD36* mRNA content in HR_{mini}, HR_{normal}, and control mice.

Fatty acid uptake capacity, with respect to (A.) H-FABP protein content (relative to total cytosolic protein) and (B.) mRNA levels of *FAT/CD36* (expressed corrected against *18S*, and normalized to mean control values, arbitrarily set at 1.0), in the gastrocnemius of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice. Data are presented as means ± SEM. Bars not sharing a common letter are significantly different ($p < 0.05$).

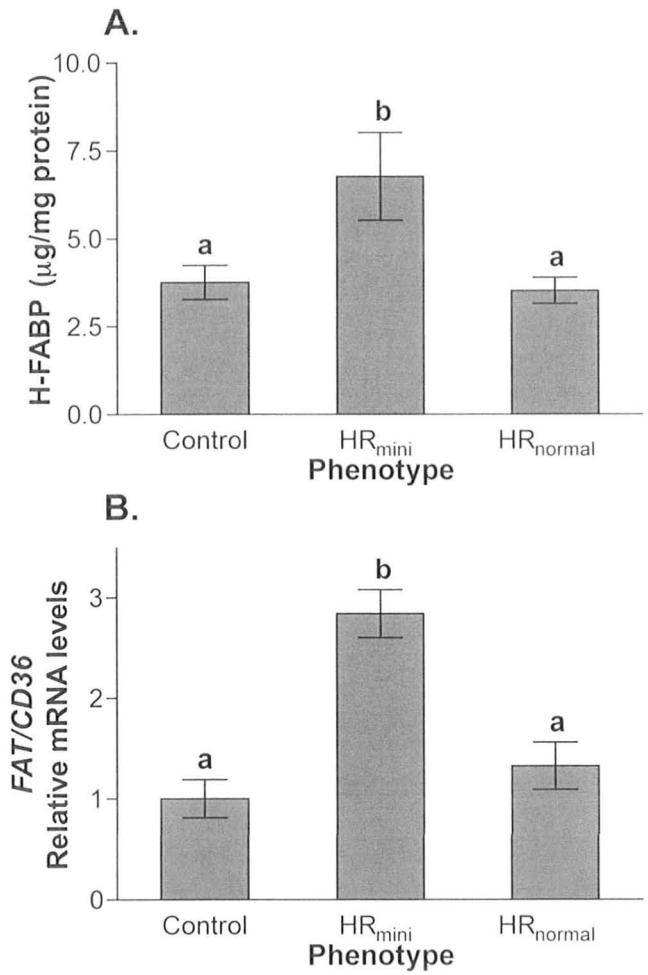

A.
H-FABP (μg/mg protein)
10.0
7.5
5.0
2.5
0.0
a
b
a
Control
HRmini
HRnormal
Phenotype
B.
FAT/CD36
Relative mRNA levels
3
2
1
0
a
b
a
Control
HRmini
HRnormal
Phenotype

Figure 2.8: Metabolic enzyme activities in the gastrocnemius of HR_{mini}, HR_{normal}, and control mice, expressed on a mass-specific and whole-muscle basis.

Maximal enzyme activities for CS, HOAD, CPT II, and HK in the gastrocnemius of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice, expressed (A.) relative to tissue wet weight (n = 8-10) or (B.) per whole muscle (n = 7-10). Data are presented as means ± SEM. For each enzyme, the presence of letters denotes statistical significance, where bars not sharing a common letter are significantly different ($p < 0.05$). U = μmol min^{-1}.

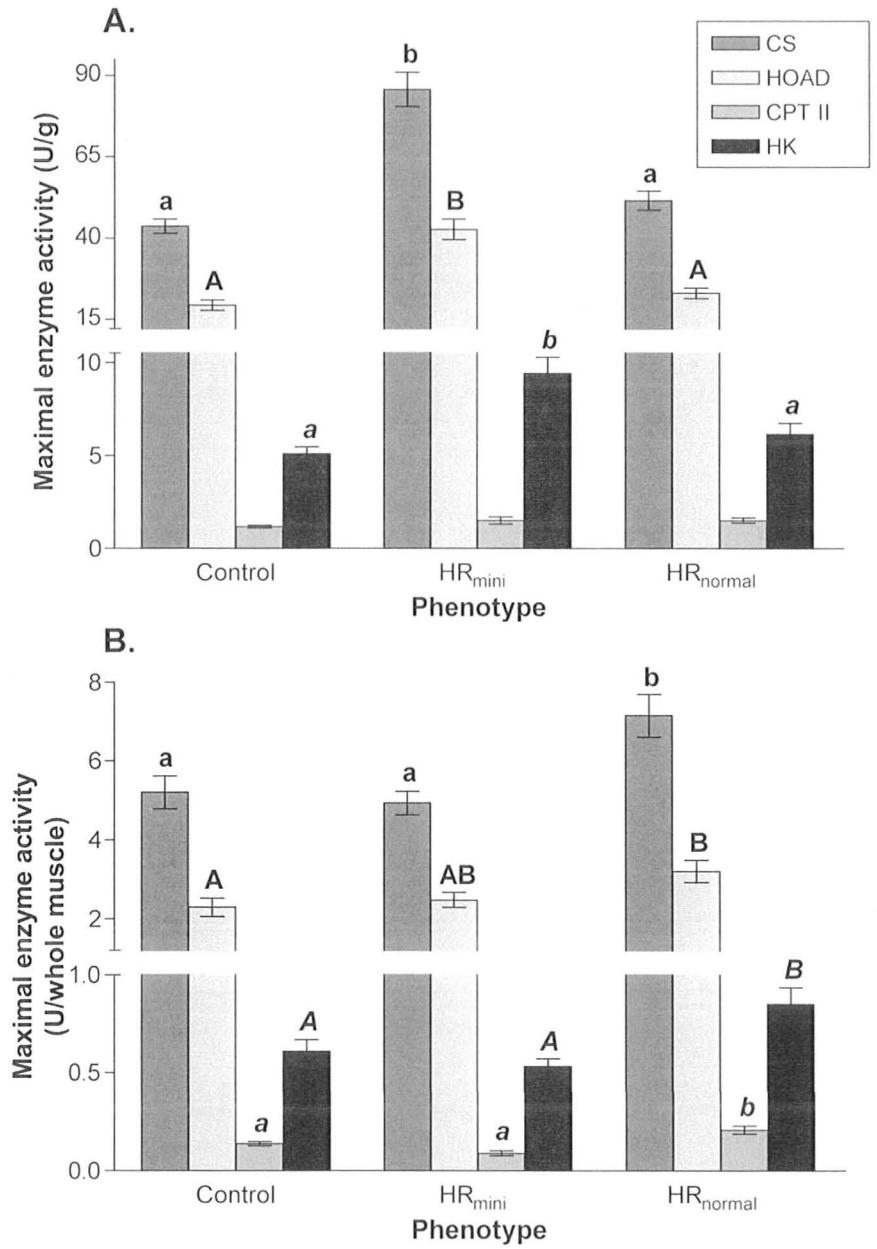
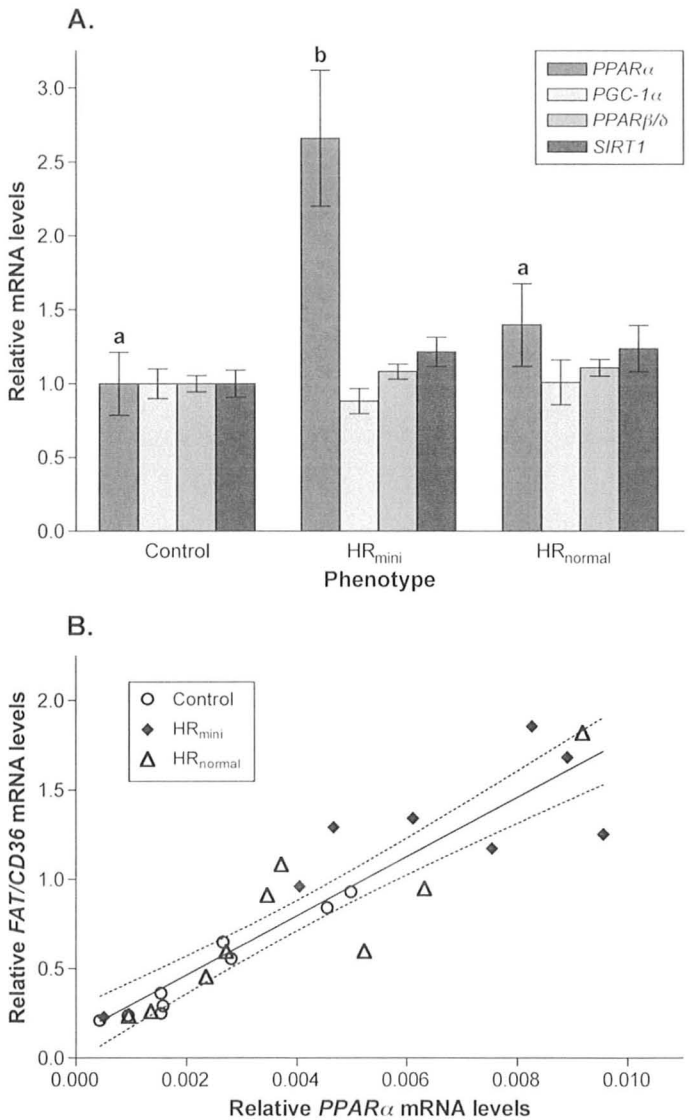

A.
CS
HOAD
CPT II
HK
Maximal enzyme activity (U/g)
90
65
40
15
10
5
0
a
A
b
B
a
A
a
b
a
Control
HRmini
HRnormal
Phenotype
B.
Maximal enzyme activity (U/whole muscle)
8
6
4
2
1.0
0.5
0.0
a
A
a
AB
b
B
a
A
a
A
b
B
Control
HRmini
HRnormal
Phenotype

Figure 2.9: mRNA levels of transcriptional factors and metabolic genes in the gastrocnemius of HR_{mini}, HR_{normal}, and control mice.

Relative gene expression of transcriptional factors in the gastrocnemius of non-selected control mice and selectively bred HR_{mini} and HR_{normal} mice (n= 7-9). In (A.), mRNA levels of transcriptional factors *PPARα*, *PGC-1α*, *PPARβ/δ*, and *SIRT1* are expressed corrected against *18S*, and normalized to mean control values (arbitrarily set at 1.0). For each gene, the presence of letters denotes statistical significance, where mouse phenotypes not sharing a common letter are significantly different ($p < 0.05$). In (B.), the relationship between the transcript levels of *PPARα* and *FAT/CD36* is presented, where mRNA levels of individual mice are expressed corrected against *18S*. The solid line represents a linear regression of all mice (all mouse phenotypes pooled), with dotted lines as 95% confidence limits. $r^2 = 0.84$, $p \leq 0.001$.


A.
b
a
a
PPARα
PGC-1α
PPARβ/δ
SIRT1
Relative mRNA levels
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
HRmini
HRnormal
Phenotype
B.
Control
HRmini
HRnormal
Relative FAT/CD36 mRNA levels
2.0
1.5
1.0
0.5
0.0
0.000
0.002
0.004
0.006
0.008
0.010
Relative PPARα mRNA levels

Discussion

It seems that it would be advantageous to adjust the mix of fuels sustaining exercising muscles to suit adaptive alterations. For instance, while carbohydrates can support high rates of ATP production and anaerobic metabolism, lipids constitute the majority of a mammal's energy stores, and are thereby the most appropriate fuel for endurance locomotion (Weber and Haman, 2004). However, I demonstrated that despite changes in voluntary locomotion, aerobic capacity, and skeletal muscle properties in response to selective breeding for high locomotor activity, there were no significant differences in the proportional mix of lipids and carbohydrates supporting ATP demand across a range of relative exercise intensities for HR_{mini}, HR_{normal}, and control mice.

Rather, absolute rates of fuel oxidation were increased in selected HR lines commensurate with changes in aerobic capacity. This is consistent with the notion that there is a conservation of a fuel use pattern amongst diverse mammals ranging in aerobic capacities, wherein relative exercise intensity dictates the proportionate contribution of carbohydrates and lipids to energy supply (McClelland, 2004). In other words, absolute rates of fuel use scale with differences in aerobic capacity in the mammals evaluated to date, including mice selectively bred for high locomotor activity. This model of experimental evolution was ideal to test the hypothesis that animals selected for increased voluntary locomotion or exhibiting an enhanced mass-specific aerobic capacity in skeletal muscle would have a greater proportionate reliance on lipids during exercise. However, it was also useful for an examination of alternate mechanisms used to increase absolute lipid oxidation rates in divergent HR lines.

Aerobic capacity and cardiac properties

As early as generation ten of selective breeding for high locomotor activity, an increased aerobic capacity (VO_{2max}, per g body mass) was observed in selected HR lines, compared to non-selected control lines (Rezende et al., 2006; Rezende et al., 2006b; Swallow et al., 1998b). Here I confirm that there is a 10-14% rise in VO_{2max} in HR mice (generation 58), but I also show that relative to body mass, male HR_{mini} mice achieve a greater VO_{2max} than HR_{normal} mice (Fig. 2.1). This had been detected in generation 36 females, but only under hypoxic conditions (Rezende et al., 2006).

It was previously demonstrated that the physiological constraints of VO_{2max} likely differ between control and HR mice – specifically, that control mice might be limited to a greater degree by central factors in the oxygen cascade (Rezende et al., 2006). Furthermore, Rezende and colleagues proposed that capacity for circulatory oxygen delivery might contribute to this variation between control and HR mice (Rezende et al., 2006a; Rezende et al., 2006). As cardiac output is an important component of oxygen delivery, we characterized some physical and metabolic properties of HR_{mini}, HR_{normal}, and control mouse hearts.

Allometric scaling of organs in the cardiorespiratory system is a key central determinant of an animal's VO_{2max} (Bishop, 1999). In fact, maximal cardiac output may be the primary limiting factor of VO_{2max}, at least in humans (Bassett Jr. and Howley, 2000). Interestingly, HR_{mini} mice have a significantly greater left ventricle mass and thus

whole heart mass than both control and HR_{normal} mice, relative to body mass (Table 2.2; consistent with the findings of Rezende et al., 2006a). However, without body mass as a covariate, neither left ventricles nor whole hearts are significantly larger in HR_{mini} mice, and left ventricles of all mouse phenotypes contain comparable levels of DNA (both on a per g wet weight and per whole ventricle basis; Table 2.2). Therefore, this difference in relative heart size seems to be predominately due to the reduced body masses of HR_{mini} mice, as opposed to the increased cardiac cell size or quantity that would be expected with hypertrophy. However, relative heart mass can give a good indication of an animal's adaptive ability to perform sustained aerobic locomotion, and there is a strong relationship between relative heart mass and VO_{2max} in birds and mammals (Bishop, 1997; Weibel et al., 2004). It is therefore likely that while HR_{mini} mice do not have significantly larger hearts *per se*, the increased ratio of heart to body size contributes to their elevated VO_{2max} by increasing oxygen and fuel delivery to working locomotor muscles. This allometric divergence may have emerged in HR_{mini} mice as a direct response to selective breeding for high locomotor activity.

Additionally, both phenotypes of HR mice show evidence of metabolic alterations in the left ventricle that are independent of phenotypic plasticity, as these changes are in sedentary mice without prior exposure to running wheels. In this first report of significant metabolic changes in the hearts of HR mice, I show that there is increased maximal activity of the glycolytic enzyme HK in the left ventricles of sedentary HR mice, compared to control mice (Fig. 2.4). This is also a feature of physiologically hypertrophic hearts in exercise-trained rats (Ji et al., 1987), as a component of the elevated overall cardiac metabolic capacity induced by exercise (see Booth and Thomason, 1991). However, in HR_{mini} mice this increased capacity for carbohydrate utilization is concurrent to a reduction in the mass-specific maximal activity of CPT II, an enzyme in the fatty acid metabolic pathway. A trend suggests the same change in HR_{normal} mice ($p = 0.100$; Fig. 2.4). This is unusual, as adult mammalian hearts predominately utilize fatty acids, and the sustained switch in preferred fuel substrate from fatty acids to carbohydrates (referred to as a reversion to the fetal phenotype) is often regarded as a deleterious hallmark of pressure- or volume-overloaded hypertrophic hearts (reviewed in Rajabi et al., 2007).

Metabolic remodeling in the heart is not well understood. It has been suggested that reversible switches in cardiac fuel substrate preference could be compensatory in some situations (Essop, 2007; Sharma et al., 2004), and there appears to be a dissociation between the physical and metabolic cardiac remodeling which occurs under environmental conditions that place pressure- and/or volume-overloading stress on hearts (Templeman et al., 2010). Furthermore, there were no significant differences in ventricular mRNA levels of several genes involved in fatty acid catabolism, including *PPARα*, *MCAD*, and muscle- and liver-type *CPT-1*, between the mouse phenotypes (Table 2.3). Thus, slightly lowering the maximal activity of CPT II (but not of another fatty acid catabolic enzyme, HOAD) in the left ventricles of HR_{mini} mice could potentially be an energy-saving mechanism. Specifically, they may be reducing the energetic "costs" of maintaining an enzyme in excess, without significantly diminishing flux through the fatty acid pathway or altering fuel preference of the heart.

I thereby propose that differences in cardiac metabolic enzyme activities in HR mouse hearts are indicative of potentially improved energetic efficiency; in conjunction with the increased relative heart size of HR_{mini} mice, these metabolic changes could contribute to the enhancement of VO_{2max} and capacity for sustained aerobic exercise in selected HR mice. Since the hearts of HR mice are ultimately capable of supporting a greater maximal aerobic capacity than control mice, and moreover, since HR mice seem to be less constrained by central factors in the oxygen transport pathway (*e.g.* cardiac output) than control mice (Rezende et al., 2006a; Rezende et al., 2006), it is highly unlikely that the cardiac metabolic remodeling in sedentary HR mice represents a deleterious reversion to the fetal phenotype.

Whole-body fuel use during exercise

To maintain ATP homeostasis when faced with increased energetic demand, it is important for an organism to make adjustments to the proportionate mixture of fuels contributing to total oxidation, in addition to increasing total flux of substrates through metabolic pathways (Weber and Haman, 2004). Contradictory to my initial hypothesis, there were no statistically significant differences in the mix of metabolic fuels across a range of submaximal relative exercise intensities for HR_{mini}, HR_{normal}, and control mice, when fuel oxidation rates were expressed as a percentage of total VO_2. Therefore, while these mouse phenotypes have diverged physiologically with respect to maximal aerobic capacity, cardiac properties that may influence VO_{2max}, and numerous skeletal muscle characteristics, these differences do not appear to have induced notable changes in the whole-body pattern of fuel selection during exercise.

During low intensity exercise (66% VO_{2max}), HR_{mini}, HR_{normal}, and control mice primarily rely on lipid oxidation for energy supply. There is a higher absolute rate of lipid oxidation (*i.e.* enhanced total lipid flux) in both HR lines, compared to control mice, despite no significant differences in the flux of carbohydrates (Figs. 2.2 and 2.3). When exercising at a higher intensity (78% VO_{2max}), the increased energetic demand in all mice is supported by an elevated flux of carbohydrates, while the absolute rate of lipid oxidation in control mice matches the rates in HR mice (Figs. 2.2 and 2.3). This suggests that HR mice do not have a markedly enhanced capacity to utilize lipids during high intensity exercise, or that it is not physiologically optimal to rely on lipids to support high intensity exercise, even if the capacity to use them at high rates exists.

Although the underlying explanation for unity of fuel use patterns in mammals has not been determined, the contribution of carbohydrates and lipids to whole-body energy supply may be influenced by numerous factors, including muscle fiber recruitment, the endocrine system, substrate availability, oxygen levels, and coordinated regulation of enzyme and substrate transporter activities and amount (Brooks, 1998; McClelland et al., 1998; McClelland, 2004). I investigated several of these variables to identify potential mechanisms used by HR mice to increase capacity for fuel oxidation, in order to reveal some alternate means by which diverse mammals might adjust metabolic flux without deviation from a conserved pattern of proportionate fuel mix during exercise.

Intramuscular glycogen stores and exercise-induced depletion

Intramuscular glycogen depots are crucial for high intensity exercise when an increasing reliance on carbohydrate metabolism is constrained at the level of circulatory glucose uptake (Vock et al., 1996a; Weber et al., 1996b); complete depletion of intramuscular glycogen stores is associated with the limits of endurance exercise and the threshold of exhaustion (Felig and Wahren, 1975). Pre-exercise intramuscular glycogen stores are therefore traditionally recognized as an indicator of an animal's endurance capacity and ability for sustained locomotion, although the relative importance of these stores may be somewhat diminished in rodents (in favor of an increased reliance on liver glycogen; Baldwin et al., 1973; Pederson et al., 2005). Male HR_{mini} mice have significantly greater mass-specific glycogen content in the gastrocnemius and plantaris (Fig. 2.5), as previously detected in the gastrocnemius of female HR_{mini} mice (Gomes et al., 2009). HR_{mini} mice are thereby able to store an equivalent whole-muscle quantity of glycogen as HR_{normal} and control mice, in hindlimb muscles that are much smaller (Fig. 2.6). This is particularly interesting considering that these "mini muscles" have a decreased proportion of type IIB fibers (Bilodeau et al., 2009; Guderley et al., 2006; Guderley et al., 2008), the fiber type which predominately relies on intramuscular glycogen for anaerobic glycolysis and tends to contain a high fraction of total muscle glycogen stores (Burke et al., 1971; Peter et al., 1972).

Aside from the enhanced mass-specific glycogen storage in several hindlimb muscles of HR_{mini} mice, there were striking dissimilarities between mouse phenotypes in the pattern of muscle glycogen depletion following a 30 minute exercise bout at a common relative intensity of 74% VO_{2max} (Figs 2.5 and 2.6). These differences suggest several interpretations that are not necessarily irreconcilable. First of all, glycogen depletion due to exercise gives an indirect indication of muscle activity, and therefore also of muscle fiber recruitment (Armstrong and Laughlin, 1985; Baldwin et al., 1973). The pattern of glycogen depletion in the soleus shows enhanced recruitment of this oxidative muscle in HR_{mini} and control mice, in contrast to HR_{normal} mice. Therefore, HR_{normal} mice may be placing less energetic demands on the soleus due to increased recruitment of faster-twitch glycolytic fibers in synergistic muscles, like the gastrocnemius (see Roy et al., 1991). There is a trend (although not statistically significant) of exercise-induced glycogen depletion in the gastrocnemius of HR_{normal} mice.

Similarly, post-exercise glycogen depletion in the tibialis anterior and plantaris suggests that under this exercise regime, HR_{normal} and control mice are recruiting glycolytic fibers in these muscles, which normally contain a high proportion of type IIB fibers (Armstrong and Phelps, 1984; Hamalainen and Pette, 1993), whereas in HR_{mini} mice these same muscles do not have a measurable quantity of type IIB fibers available for recruitment (Bilodeau et al., 2009; Guderley et al., 2006). Although the tibialis anterior muscle is composed entirely of type IIA and IID muscle fibers in the HR_{mini} mice (Bilodeau et al., 2009), and these oxidative muscle fibers tend to have a lower recruitment threshold than type IIB fibers (Armstrong and Phelps, 1984; Armstrong and Laughlin, 1985; Hamalainen and Pette, 1993), HR_{mini} mice did not show significant glycogen

depletion in the tibialis anterior after the bout of exercise.

Preservation of muscle glycogen depots may signify that during this moderately high intensity exercise HR_{mini} mice do not place as much physical demand on the tibialis anterior muscle as the other mice. However, there is also a strong possibility that there is a greater dependence on aerobic metabolism in HR_{mini} mice, thus sparing some muscle glycogen reserves. Muscles with a higher proportion of oxidative fibers tend to show minimal glycogen depletion during prolonged contractile activity, likely because they are supported by a greater capacity for fatty acid oxidation (Baldwin and Tipton, 1972). The hindlimb muscles of HR_{mini} mice have been shown to contain reduced glycogen phosphorylase (both the activated form and total content) on a whole muscle basis, which further supports the proposed sparing of intramuscular glycogen stores during exercise (Houle-Leroy et al., 2003).

Nonetheless, HR_{mini} mice do not have a significantly reduced rate of carbohydrate utilization compared to the other mice, under a common exercise regime. Thus, perhaps they are more dependent on circulatory glucose to achieve a comparable degree of carbohydrate metabolism as mice that are recruiting type IIB fibers and placing a greater reliance on muscle glycogen stores. Indeed, liver glycogen appears to be a more crucial source of energy than muscle glycogen during exercise in rodents (Baldwin et al., 1973; Pederson et al., 2005). Resting HR_{mini} mice (without prior wheel access) have larger liver glycogen reserves than the other mouse phenotypes, and with wheel access, all HR mice have augmented protein levels of the glucose transporter GLUT-4 in the gastrocnemius, relative to control mice (Gomes et al., 2009). HR mice also show enhanced insulin-stimulated glucose uptake in the extensor digitorum longus muscle, irrespective of wheel access (Dumke et al., 2001). Furthermore, HR_{mini} mice have a higher capillary-to-fiber ratio and higher capillary density in the gastrocnemius, compared to mice with the normal muscle phenotype (Wong et al., 2009). An increased capillarity of hindlimb muscles is likely to play an important role in enhancing utilization of circulatory fuels in HR_{mini} mice, as structural constraints in the delivery of exogenous fuels may factor in the greater dependence on intramuscular fuels that occurs with increasing exercise intensity in mammals (Vock et al., 1996b; Weber et al., 1996b).

Previous experiments have highlighted differences between distinct mammals in the preferred source of substrates metabolized by working muscle. In an example of similar sized mammals differing in aerobic capacity, dogs appear to have a relatively greater dependence on intramuscular glycogen and triacylglyceride stores than goats; despite wide divergence in whole-animal metabolic rates, the maximal rates of circulatory fuel oxidation are only slightly different between these animals (Vock et al., 1996a; Weber et al., 1996a; Weber et al., 1996b). However, these studies used phylogenetically distant species, so it is unclear if inter-animal differences were solely due to differences in aerobic capacity. It would be valuable to empirically determine the partitioning of intramuscular versus circulatory fuel sources during exercise in selectively bred HR mice and non-selected controls.

Numerous discrepancies in experimental design preclude a direct comparison of my glycogen results with those of Gomes and colleagues, whose experiments involved voluntary wheel-running, variable exercise intensities and running duration, previous

wheel exposure, fed mice, females, and the confounding effect of circadian glycogen rhythms (Gomes et al., 2009). Nonetheless, it is apparent that in both studies there was a distinct effect of the mini muscle phenotype on muscle glycogen depletion patterns. Although it is unfeasible to make definitive conclusions about hindlimb muscle recruitment patterns without glycogen measurements for every muscle, my results show that at a common relative exercise intensity, HR_{mini} mice differ from HR_{normal} and control mice in their utilization of various muscles and fiber types. I propose that compared to mice with the normal muscle phenotype, HR_{mini} mice have a greater reliance on oxidative fibers and aerobic metabolism, as well as an increased dependence on circulatory (rather than intramuscular) fuels. Intriguingly, these results insinuate that the mammalian whole-body fuel use pattern can be maintained despite differential muscle fiber recruitment patterns. This is incongruent to the observation that the pattern of whole-body fuel use tends to match progressive muscle recruitment, where increasing reliance on carbohydrates at higher exercise intensities occurs in conjunction with increasing recruitment of glycolytic muscle fibers (McClelland, 2004; Roberts et al., 1996).

Fuel uptake into working muscle

In further support of the proposed increased utilization of exogenous glucose in skeletal muscle, HR_{mini} mice have enhanced mass-specific maximal HK activity in the gastrocnemius (Fig. 2.8A). HK phosphorylates glucose to generate glucose-6-phosphate, and in doing so is an essential coordinator of glucose uptake into the myocyte, as this reaction serves to maintain the downhill glucose gradient from the blood (Halseth et al., 1999). Transgenic mice overexpressing HK II show increased glucose uptake into skeletal muscle during moderate-intensity exercise (Halseth et al., 1999), and in a similar manner increased mass-specific maximal HK activity in the gastrocnemius of HR_{mini} mice may enable enhanced glucose uptake from the circulatory system (see also Bilodeau et al., 2009; Guderley et al., 2006; Houle-Leroy et al., 2003). HK activity is an especially important controlling factor for regulating glucose uptake during exercise in more oxidative muscles (Fueger et al., 2003), such as those of HR_{mini} mice.

HR_{mini} mice also have an increased capacity for fatty acid uptake into working muscles. Due to an 80% higher cytosolic content of H-FABP (Fig. 2.7A), a protein which serves as an intracellular sink for fatty acids transported across the sarcolemma and facilitates their movement through the cytoplasm (Glatz et al., 2003), it is likely that the "mini muscle" gastrocnemius has a greater capability to increase flux of exogenous fatty acids into the muscle than the normal muscle phenotype. This concurs with the observation that muscles with a higher proportion of oxidative fibers tend to have a higher cytosolic content of H-FABP (van Nieuwenhoven et al., 1995; Vork et al., 1991). Although it seems that H-FABP is not a direct regulator of fatty acid translocation across the sarcolemma (on account of its abundance in the cytoplasm, it has a permissive, albeit important, role in muscular fatty acid uptake), increased H-FABP levels could accommodate rapid changes in substrate flux, such as during the transition from resting to contracting muscle (Luiken et al., 2003).

Furthermore, HR_{mini} mice show greatly enhanced gastrocnemius gene expression of fatty acid translocase, *FAT/CD36*, which is a pivotal sarcolemmal transporter of long-chain fatty acids (Fig 2.7B). Overexpression of FAT/CD36 in rat skeletal muscle leads to elevated fatty acid oxidation, in addition to increased rates of fatty acid transport (Nickerson et al., 2009). It seems likely that increasing the muscle protein content of FAT/CD36 would be an important mechanism for altering the whole-body fuel use, considering that this fatty acid transporter may facilitate as much as 50% of fatty acid uptake in muscle (Luiken et al., 1999). Although increased gene transcription of *FAT/CD36* does not mean that protein content of this fatty acid transporter is also elevated, it is plausible that in the gastrocnemius of HR_{mini} mice there may be more FAT/CD36 protein available for translocation to the sarcolemma during periods of acute demand for increased fatty acid uptake (*i.e.* during exercise).

These changes implicating an increased capacity for fatty acid uptake into the skeletal muscle of HR_{mini} mice are pertinent in light of the fact that my *in vivo* results suggest that these mice do not have (or do not capitalize upon) an enhanced whole-body ability to utilize lipids during high intensity exercise. This implies that fatty acid uptake from circulation is not the sole factor restricting lipid oxidation during high-intensity exercise.

Skeletal muscle capacity for oxidative metabolism

The increased capacity for fatty acid uptake into the skeletal muscle of HR_{mini} mice is accompanied by an enhanced mass-specific capacity to utilize fatty acids. In addition to the increased mass-specific maximal activities of HK and CS, which is a trademark of the "mini muscle" phenotype's enhanced aerobic capacity (Guderley et al., 2006; Houle-Leroy et al., 2003), I show that sedentary HR_{mini} mice also have an enhanced capacity for β-oxidation at the level of HOAD activity (Fig. 2.8A). Coordinated elevation of maximal activities of enzymes in both fatty acid and carbohydrate metabolic pathways (see also Houle-Leroy et al., 2003) supports the concept of an increased mass-specific aerobic capacity of skeletal muscle without a shift towards preferential utilization of fatty acids in HR_{mini} mice.

On a whole-muscle basis, HR_{mini} mice have equivalent maximal enzyme activities as control mice (Fig 2.8B; see also Houle-Leroy et al., 2003), with the reduced energetic costs associated with smaller gastrocnemius muscles that are resistant to fatigue (Syme et al., 2005). Conversely, HR_{normal} mice appear to exhibit an enhancement of the overall metabolic capacity of the gastrocnemius, for due to the slight muscle enlargement compared to control mice, HR_{normal} mice have greater whole-muscle maximal activities of CS, HOAD, CPT II, and HK in the gastrocnemius, compared to HR_{mini} (with the exception of HOAD) or control mice (Fig. 2.8B). This may be one contributing mechanism by which HR_{normal} mice achieve an equivalent rate of lipid and carbohydrate utilization as HR_{mini} mice (*i.e.* higher whole-muscle enzyme activities in contrast to higher mass-specific enzyme activities in smaller muscles).

Transcriptional regulation of skeletal muscles properties

The increased mass-specific HOAD activity in the HR_{mini} gastrocnemius is likely under some degree of transcriptional control, as there are significantly elevated mRNA levels of *PPARα*, compared to control or HR_{normal} mice (Fig. 2.9A). PPARα is an important transcriptional regulator of a series of genes that includes HOAD (Zhang et al., 1992). Changing the expression of metabolic enzyme genes is one putative mechanism for chronically altering flux through a metabolic pathway, and thus influencing whole-body fuel use patterns (McClelland, 2004).

Elevated *PPARα* expression may in fact be an underlying basis for multiple altered metabolic characteristics of the "mini muscle." In skeletal muscle, PPARα is a predominant isoform of the PPAR family of transcriptional regulators; upon activation PPARα is involved in the regulation of many genes in the fatty acid metabolic pathway (see McClelland, 2004). My results show a very strong correlation between mRNA levels of *PPARα* and *FAT/CD36* amongst all mice (Fig. 2.9B), which suggests a close relationship between the transcript levels of these two genes. Indeed, it has been shown that PPARα-activating drugs induce gene expression of *FAT/CD36* (Motojima et al., 1998), and the murine *FAT/CD36* gene promoter may contain two peroxisome-proliferator-response elements (PPREs), which are the specific response elements through which PPARs regulate gene transcription (Teboul et al., 2001).

It is improbable that the increased mRNA levels of *PPARα* and *FAT/CD36* in the gastrocnemius of HR_{mini} muscles is an effect of differential gene dosage, because gene expression of *PGC-1α*, *PPARβ/δ*, and *SIRT1* are not significantly different between HR_{mini}, HR_{normal}, or control mice (Fig. 2.9A). These factors are involved in coordinating various cellular responses, including the enhancement of PPARα binding to DNA by the transcriptional coactivator PGC-1α (Vega et al., 2000), the expression of genes associated with mitochondrial biogenesis and fatty acid oxidation by PPARβ/δ (Wang et al., 2004), and the coupling of energy requirements to transcriptional regulation by the NAD-dependent deacetylase SIRT1 (see Freyssenet, 2007). Increased activation of PGC-1α (Lin et al., 2002) or PPARβ/δ (Luquet et al., 2003; Wang et al., 2004) has been implicated in the induction of muscle fiber type switching (*i.e.* transition into more oxidative fiber types). In addition, SIRT1 has been shown to be an important functional regulatory of PGC-1α, and to be crucial for the upregulation of fatty acid oxidation under certain conditions (Gerhart-Hines et al., 2007). However, based on unchanged mRNA levels amongst all mice in this study, it does not seem likely that these transcriptional factors play a major role in altered characteristics of the "mini muscle."

Summary and concluding remarks

Selective breeding for high levels of voluntary locomotion resulted in an increased aerobic capacity amongst all HR mice, relative to non-selected controls, with HR_{mini} mice demonstrating a further elevation in mass-specific VO_{2max} above HR_{normal} mice. This enhancement of aerobic capacity might be partially supported by allometric adjustments resulting in a greater relative heart mass in the HR_{mini} mice, and changes in the maximal

activities of cardiac metabolic enzymes in HR mouse hearts. However, despite divergent patterns in exercise behavior and aerobic capacity, there are no statistically significant differences in the whole-body pattern of proportionate fuel use across a range of relative exercise intensities for HR_{mini}, HR_{normal}, and control mice. In selected HR mice, absolute fuel oxidation rates are greater, commensurate with increases in aerobic capacity. By investigating some of the physiological factors thought to be instrumental in dictating whole-body fuel use, I demonstrated that phenotypically diverse HR mice maintain an apparently conserved fuel use pattern via alternate mechanisms for enhancing flux through metabolic pathways.

Under a common exercise regime, HR_{mini} mice differ from HR_{normal} and control mice with respect to muscle recruitment, suggesting that HR_{mini} mice might place a greater dependence on oxidative muscle fibers and aerobic metabolism, as well as a potentially increased reliance on circulatory (rather than intramuscular) carbohydrate supply. Furthermore, compared to mice with a normal muscle phenotype, HR_{mini} mice may have an increased capacity for fatty acid uptake into exercising muscle, at the level of mRNA content of trans-sarcolemmal transporter *FAT/CD36* and cytosolic H-FABP protein content. This is concurrent to an enhanced mass-specific capacity for fatty acid utilization (evidenced by increased maximal activities of HOAD and CS) and circulatory glucose uptake (at the level of maximal HK activity). Gastrocnemius *PPARα* mRNA levels are substantially greater than those of HR_{normal} or control mice, and are closely correlated with *FAT/CD36* mRNA levels, which implies that this transcriptional regulator likely plays an important role in mediating the metabolic differences in the "mini muscle" phenotype. HR_{normal} mice on the other hand seem to have evolved with divergent mechanisms from HR_{mini} mice for enhancing muscle oxidative capacity, perhaps partially by increasing whole-muscle metabolic enzyme activities.

It is notable that these skeletal muscle differences between sedentary HR_{mini}, HR_{normal}, and control mice are innate, rather than resulting from exercise-induced phenotypic plasticity. Consequently, this animal model highlights numerous physiological traits involved in the mediation of whole-body fuel use that can be divergently altered during the evolution of a mammal with enhanced voluntary locomotor activity and aerobic capacity. These include some features previously postulated to be conserved amongst diverse mammals, such as the pattern of muscle fiber recruitment during exercise (see McClelland, 2004; Roberts et al., 1996) and the limitations of circulatory fuel supply during high intensity exercise (see Vock et al., 1996b; Weber et al., 1996a; Weber et al., 1996b). Thus, it seems that there are multiple mechanisms to enhance fuel oxidation rates with the development of an elevated aerobic capacity, and mammals can differentially utilize these mechanisms without deviating from a conserved whole-body pattern of fuel use. Plastic or adaptive alterations to all or any of these physiological factors likely contribute to the underlying means by which genetically and phenotypically distinct mammals retain a conserved pattern of fuel selection during exercise.

CHAPTER 3: GENERAL SUMMARY AND CONCLUSIONS

In this study, I demonstrated that despite changes in voluntary locomotion, aerobic capacity, and skeletal muscle properties in response to selective breeding for high locomotor activity, there were no significant differences in the proportional mix of lipids and carbohydrates supporting ATP demand across a range of relative exercise intensities for HR_{mini}, HR_{normal}, and control mice (Figs. 2.1-2.3).

HR mice primarily achieve greater levels of voluntary locomotion by running faster than control mice on the running wheel (Garland et al., 2002; Garland et al., 2010; Girard et al., 2001; Swallow et al., 1998a). My findings suggest that due to an increased aerobic capacity, HR mice are able to run at these faster speeds while still predominately sustained by lipid oxidation. In contrast, control mice would need to utilize a higher proportion of carbohydrates to achieve equivalent absolute exercise intensities. Therefore, although selective breeding did not alter fuel preference in the sense that there was shift towards a greater proportion of lipids contributing to total oxidation at all relative exercise intensities, I believe that metabolic fuel use was an important trait in this selective regime. An enhanced capacity for lipid oxidation in conjunction with an increased VO_{2max} may have enabled the shift in locomotor speed preference in HR mice, thus allowing them to achieve greater levels of voluntary locomotion, for which they are selectively bred.

This is consistent with observations made by Roberts and colleagues: that dogs "choose" (or have the capacity for) higher locomotor speeds and endurance in their natural habits than more sedentary goats, but as these diverse animals range in aerobic capacity, both are predominately utilizing lipids during the low-to-moderate relative exercise intensities of everyday locomotion (Roberts et al., 1996). Therefore, my study has confirmed one of the major findings of these comparative experiments, using an experimental evolution approach. It appears that mammals can achieve diversity in locomotor behavior through adaptive adjustments to both aerobic capacity and lipid oxidative capacity. This may be an underlying explanation for evident unity in fuel use patterns amongst mammals ranging in aerobic capacities, when absolute rates of oxidation are scaled to VO_{2max}.

However, my investigation also indicates that mammals can diverge in their mechanisms for enhancing fuel oxidation rates. In the case of selectively-bred high running mice, HR_{mini} and HR_{normal} mice differ with respect to their muscle fiber recruitment patterns during exercise, capacity for fatty acid uptake into skeletal muscle, metabolic enzyme activities (*i.e.* higher mass-specific activities in smaller muscles contrasting with greater whole muscle activities due to muscle enlargement), and gene expression of *PPARα*, a transcriptional regulator of fatty acid metabolic pathways (Figs. 2.5-2.9).

Notably, some of the metabolic regulatory mechanisms that may diverge between HR_{mini} and HR_{normal} mice have previously been suggested to be pervasive amongst mammals, thus potentially contributing to the unity in fuel use patterns. For instance, the

progression of muscle fiber recruitment has been observed to match whole-body fuel use, with the increasing proportionate reliance on carbohydrates at higher exercise intensities corresponding to the recruitment of fast glycolytic muscle fibers (McClelland, 2004; Roberts et al., 1996). However, my investigation demonstrates that mammals may differ in their muscle recruitment patterns during exercise, and yet maintain an apparently conserved pattern of fuel selection. It is particularly intriguing that HR_{mini} mice do not seem to have type IIB muscle fibers in hindlimb muscles available for recruitment, but they still show an equivalent proportional reliance on carbohydrates for total oxidation at higher exercise intensities.

It has also been suggested that structural constraints at the level of trans-sarcolemmal transport might be an important limiting factor in the utilization of circulatory substrates for mammals, particularly at higher exercise intensities (Vock et al., 1996a; Weber et al., 1996a; Weber et al., 1996b). It seems logical that animals selectively bred to exhibit increased locomotion would have adaptations to enable augmented utilization of exogenous fuel reserves, since the quantity of fuel that can be stored intramuscularly is limited and could not exclusively sustain exercise for a prolonged duration (McClelland et al., 1994). However, despite no differences in fatty acid utilization, HR_{mini} mice have a greater capacity for using circulatory sources of metabolic fuels compared to HR_{normal} mice, at least on a mass-specific basis. This is evidenced by their higher cytosolic H-FABP content, increased transcript levels of *FAT/CD36*, an enhanced mass-specific capacity for glucose phosphorylation by HK, and sparing of intramuscular glycogen stores in the tibialis anterior muscle (Figs. 2.5-2.8A). HR_{normal} mice appear to have alternate means to elevate metabolic flux above control mice, and this may not include an increased dependence on circulatory fuel supply.

Based on this investigation, it seems unlikely that the proportional contribution of lipids to total oxidation is constrained at one crucial step that is common for all mammals. Rather, total lipid flux appears to be regulated by multiple integrated factors, and the relative importance of each of these factors can vary amongst genotypically and phenotypically diverse mammals.

Future directions

My study presents a number of implications that could be further validated using this model of experimental evolution. For instance, it would be valuable to empirically determine the partitioning of intramuscular versus circulatory fuel sources during exercise in these mice. This could be done through continuous infusions of isotopically labeled glucose or palmitate, indirect calorimetry, and measurements of intramuscular fuel substrates depletion due to exercise. By using all eight lines of selectively bred HR mice and non-selected controls, these experiments could provide some indication of whether there are conserved aspects of fuel partitioning amongst "high running" or aerobic mammals during exercise.

Furthermore, this animal model could be utilized to test the importance of various regulatory steps in the fatty acid metabolic pathway. For instance, if HR_{mini} mice have increased protein content of FAT/CD36 compared to mice with the normal muscle phenotype, it could be determined whether upregulation of this fatty acid transporter is a

crucial adaptation for HR_{mini} mice to achieve enhanced mass-specific fatty acid oxidation. This could be assessed through a series of *in vitro* experiments in which FAT/CD36 activity is blocked by the specific inhibitor sulfo-N-succimidyl oleate (SSO).

Lastly, it would be of interest to evaluate the female mice in this animal model. There appears to be a difference between males and females in terms of fuel use patterns, with females exhibiting higher maximal rates of lipid oxidation and an increased proportional contribution of lipids to energy expenditure across a range of relative exercise intensities, compared to men (Friedlander et al., 1998; Tarnopolsky et al., 1990; Venables et al., 2005). Furthermore, there is a sex-specific component of locomotor behavior in selectively bred mice, as HR males spend more time running than control males, in addition to running at higher speeds; HR females simply run faster and more intermittently than control females (Garland et al., 2010; Girard et al., 2001; Rezende et al., 2009). This is also evident in their cage activity without access to wheels: during nighttime activity, HR females exhibit increased utilization of carbohydrates compared to control females (suggestive of increased burst exercise), whereas HR males appear to have slightly higher rates of fatty acid oxidation, compared to control males (Jónás et al., 2010). Therefore, this model of experimental evolution could potentially be used to tease out some of the mechanistic explanations for differences between males and females in the fuel use pattern during exercise.

My study highlights some innate subordinate traits that may contribute to the high locomotor activity in selectively bred HR mice. Specifically, in the HR lines tested, increasing capacity for lipid oxidation in conjunction with increasing aerobic capacity might be an important component of attaining higher levels of voluntary locomotion. Metabolic alterations such as increased content of H-FABP cytosolic protein, *FAT/CD36* mRNA, and *PPARα* mRNA, in addition to the enhanced activities of aerobic enzymes, may thereby provide the selective benefit of the "mini muscle" phenotype.

Moreover, by demonstrating that HR_{mini} mice and HR_{normal} mice have evolved with alternate means of achieving an increased capacity for lipid oxidation, this investigation has furthered our understanding of the mammalian fuel use pattern during exercise. There are multiple mechanisms to enhance fuel oxidation rates with the development of an elevated aerobic capacity, and mammals can differentially utilize these mechanisms without deviating from a conserved whole-body pattern of fuel use.

REFERENCES

Aoyama T, Peters JM, Iritani N, Nakajima T, Furihata K, Hashimoto T, Gonzalez FJ. 1998. Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor α (PPARα). *J Biol Chem* 273:5678-5684.

Armstrong RB, Phelps RO. 1984. Muscle fiber type composition of the rat hindlimb. *Am J Anat* 171:259-272.

Armstrong R, Laughlin M. 1985. Metabolic indicators of fibre recruitment in mammalian muscles during locomotion. *J Exp Biol* 115:201-213.

Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F, Mostoslavsky R, Alt FW, Schibler U. 2008. SIRT1 regulates circadian clock gene expression through PER2 deacetylation. *Cell* 134:317-328.

Baldwin KM, Tipton CM. 1972. Work and metabolic patterns of fast and slow twitch skeletal muscle contracting *in situ*. *Pflüg Archiv Eur J Physiol* 334:345-356.

Baldwin K, Reitman J, Terjung R, Winder W, Holloszy J. 1973. Substrate depletion in different types of muscle and in liver during prolonged running. *Am J Physiol* 225:1045-1050.

Bassett Jr. DR, Howley ET. 2000. Limiting factors for maximum oxygen uptake and determinants of endurance performance. *Med Sci Sports Exerc* 32:70-84.

Bergman BC, Brooks GA. 1999. Respiratory gas-exchange ratios during graded exercise in fed and fasted trained and untrained men. *J Appl Physiol* 86:479-487.

Bilodeau GM, Guderley H, Joanisse DR, Garland T,Jr. 2009. Reduction of type IIb myosin and IIB fibers in tibialis anterior muscle of mini-muscle mice from high-activity lines. *J Exp Zool Part A: Ecol Genet Physiol* 311A:189-198.

Binas B, Danneberg H, McWhir J, Mullins L, Clark AJ. 1999. Requirement for the heart-type fatty acid binding protein in cardiac fatty acid utilization. *FASEB J* 13:805-812.

Bishop CM. 1999. The maximum oxygen consumption and aerobic scope of birds and mammals: Getting to the heart of the matter. *Proc R Soc Lond [Biol]* 266:2275-2281.

Bishop CM. 1997. Heart mass and the maximum cardiac output of birds and mammals: Implications for estimating the maximum aerobic power input of flying animals. *Philos Trans R Soc Lond B Biol Sci* 352:447-456.

Bonen A, Luiken JJFP, Arumugam Y, Glatz JFC, Tandon NN. 2000. Acute regulation of fatty acid uptake involves the cellular redistribution of fatty acid translocase. *J Biol Chem* 275:14501-14508.

Booth FW, Thomason DB. 1991. Molecular and cellular adaptation of muscle in response to exercise: Perspectives of various models. *Physiol Rev* 71:541-585.

Brand MD. 2005. The efficiency and plasticity of mitochondrial energy transduction. *Biochem Soc Trans* 33:897-904.

Brandt JM, Djouadi F, Kelly DP. 1998. Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor α. *J Biol Chem* 273:23786-23792.

Brooks GA, Mercier J. 1994. Balance of carbohydrate and lipid utilization during exercise: The "crossover" concept. *J Appl Physiol* 76:2253-2261.

Brooks GA. 1998. Mammalian fuel utilization during sustained exercise. *Comp Biochem Physiol B Biochem Mol Biol* 120:89-107.

Burke RE, Levine DN, Zajac FE,III, Tsairis P, Engel WK. 1971. Mammalian motor units: Physiological-histochemical correlation in three types in cat gastrocnemius. *Science* 174:709-712.

Butz CE, McClelland GB, Brooks GA. 2004. MCT1 confirmed in rat striated muscle mitochondria. *J Appl Physiol* 97:1059-1066.

Cikos S, Bukovska A, Koppel J. 2007. Relative quantification of mRNA: Comparison of methods currently used for real-time PCR data analysis. *BMC Mol Biol* 8:113.

Dewsbury DA. 1980. Wheel-running behavior in 12 species of muroid rodents. *Behav Processes* 5:271-280.

Dlugosz EM, Chappell MA, McGillivray DG, Syme DA, Garland T,Jr. 2009. Locomotor trade-offs in mice selectively bred for high voluntary wheel running. *J Exp Biol* 212:2612-2618.

Dubouchaud H, Butterfield GE, Wolfel EE, Bergman BC, Brooks GA. 2000. Endurance training, expression, and physiology of LDH, MCT1, and MCT4 in human skeletal muscle. *Am J Physiol Endocrinol Metab* 278:E571-579.

Dudley R, Gans C. 1991. A critique of symmorphosis and optimality models in physiology. *Physiol Zool* 64:627-637.

Dumke CL, Rhodes JS, Garland T,Jr, Maslowski E, Swallow JG, Wetter AC, Cartee GD. 2001. Genetic selection of mice for high voluntary wheel running: Effect on skeletal muscle glucose uptake. *J Appl Physiol* 91:1289-1297.

Edwards HT, Margaria R, Dill DB. 1934. Metabolic rate, blood sugar and the utilization of carbohydrate. *Am J Physiol* 108:203-209.

Essop MF. 2007. Cardiac metabolic adaptations in response to chronic hypoxia. *J Physiol* 584:715-726.

Felig P, Wahren J. 1975. Fuel homeostasis in exercise. *N Engl J Med* 293:1078-1084.

Frayn KN. 1983. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 55:628-634.

Freyssenet D. 2007. Energy sensing and regulation of gene expression in skeletal muscle. *J Appl Physiol* 102:529-540.

Friedlander AL, Casazza GA, Horning MA, Huie MJ, Piacentini MF, Trimmer JK, Brooks GA. 1998. Training-induced alterations of carbohydrate metabolism in women: Women respond differently from men. *J Appl Physiol* 85:1175-1186.

Fueger PT, Heikkinen S, Bracy DP, Malabanan CM, Pencek RR, Laakso M, Wasserman DH. 2003. Hexokinase II partial knockout impairs exercise-stimulated glucose uptake in oxidative muscles of mice. *Am J Physiol Endocrinol Metab* 285:E958-963.

Garland T, Jr., Morgan MT, Swallow JG, Rhodes JS, Girard I, Belter JG, Carter PA, Cheverud J. 2002. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. *Evolution* 56:1267-1275.

Garland T, Jr. 2003. Selection experiments: An under-utilized tool in biomechanics and organismal biology. In: Bels VL, Gasc JP and Casinos A, editors. Vertebrate biomechanics and evolution. Oxford, U.K.: BIOS Scientific Publishers. p 23-56

Garland T, Jr., Huey RB. 1987. Testing symmorphosis: Does structure match functional requirements? *Evolution* 41:1404-1409.

Garland T, Jr., Kelly SA. 2006. Phenotypic plasticity and experimental evolution. *J Exp Biol* 209:2344-2361.

Garland T, Kelly SA, Malisch JL, Kolb EM, Hannon RM, Keeney BK, Van Cleave SL, Middleton KM. 2010. How to run far: Multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. *Proc R Soc B*

Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim S, Mostoslavsky R, Alt FW, Wu Z, Puigserver P. 2007. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1α. *EMBO J* 26:1913-1923.

Girard I, McAleer MW, Rhodes JS, Garland T, Jr. 2001. Selection for high voluntary wheel-running increases speed and intermittency in house mice (*mus domesticus*). *J Exp Biol* 204:4311-4320.

Glatz JFC, Schaap FG, Binas B, Bonen A, van der Vusse GJ, Luiken JJFP. 2003. Cytoplasmic fatty acid - binding protein facilitates fatty acid utilization by skeletal muscle. *Acta Physiol Scand* 178:367-371.

Glatz JFC, Luiken JJFP, Bonen A. 2010. Membrane fatty acid transporters as regulators of lipid metabolism: Implications for metabolic disease. *Physiol Rev* 90:367-417.

Gomes FR, Rezende EL, Malisch JL, Lee SK, Rivas DA, Kelly SA, Lytle C, Yaspelkis BB, III, Garland Jr. T. 2009. Glycogen storage and muscle glucose transporters (GLUT-4) of mice selectively bred for high voluntary wheel running. *J Exp Biol* 212:238-248.

Guderley H, Houle-Leroy P, Diffee GM, Camp DM, Garland Jr. T. 2006. Morphometry, ultrastructure, myosin isoforms, and metabolic capacities of the "mini muscles" favoured by selection for high activity in house mice. *Comp Biochem Physiol B Biochem Mol Biol* 144:271-282.

Guderley H, Joanisse DR, Mokas S, Bilodeau GM, Garland Jr. T. 2008. Altered fibre types in gastrocnemius muscle of high wheel-running selected mice with mini-muscle phenotypes. *Comp Biochem Physiol B Biochem Mol Biol* 149:490-500.

Gulick T, Cresci S, Caira T, Moore DD, Kelly DP. 1994. The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. *Proc Natl Acad Sci U S A* 91:11012-11016.

Halseth AE, Bracy DP, Wasserman DH. 1999. Overexpression of hexokinase II increases insulinand exercise-stimulated muscle glucose uptake in vivo. *Am J Physiol Endocrinol Metab* 276:E70-77.

Hamalainen N, Pette D. 1993. The histochemical profiles of fast fiber types IIB, IID, and IIA in skeletal muscles of mouse, rat, and rabbit. J Histochem Cytochem 41:733-743.

Hill AV, Lupton H. 1923. Muscular exercise, lactic acid, and the supply and utilization of oxygen. *Q J Med* 16:135-171.

Hochachka PW, Somero GN. 2002. Biochemical adaptation: Mechanism and process in physiological evolution. New York: Oxford University Press.

Houle-Leroy P, Garland T, Jr., Swallow JG, Guderley H. 2000. Effects of voluntary activity and genetic selection on muscle metabolic capacities in house mice *mus domesticus*. *J Appl Physiol* 89:1608-1616.

Houle-Leroy P, Guderley H, Swallow JG, Garland T, Jr. 2003. Artificial selection for high activity favors mighty mini-muscles in house mice. *Am J Physiol Regul Integr Comp Physiol* 284:R433-443.

Husak JF. 2006. Does survival depend on how fast you *can* run or how fast you *do* run? *Funct Ecol* 20:1080-1086.

Jeukendrup AE. 2002. Regulation of fat metabolism in skeletal muscle. *Ann N Y Acad Sci* 967:217-235.

Ji LL, Stratman FW, Lardy HA. 1987. Effects of β1- and β1 + β2-antagonists on training-induced myocardial hypertrophy and enzyme adaptation. *Biochem Pharmacol* 36:3411-3417.

Jónás I, Vaanholt LM, Doornbos M, Garland Jr. T, Scheurink AJW, Nyakas C, van Dijk G. 2010. Effects of selective breeding for increased wheel-running behavior on circadian timing of substrate oxidation and ingestive behavior. *Physiol Behav* 99:549-554.

Leonard JBK. 1999. Regional variation in muscle metabolic enzymes in individual american shad (*alosa sapidissima*). *Can J Zool* 77:1322-1326.

Lin J, Wu H, Tarr PT *et al.* 2002. Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. *Nature* 418:797-801.

Luiken J, Koonen D, Coumans W, Pelsers M, Binas B, Bonen A, Glatz J. 2003. Long-chain fatty acid uptake by skeletal muscle is impaired in homozygous, but not heterozygous, heart-type-FABP null mice. *Lipids* 38:491-496.

Luiken JJFP, Turcotte LP, Bonen A. 1999. Protein-mediated palmitate uptake and expression of fatty acid transport proteins in heart giant vesicles. *J Lipid Res* 40:1007-1016.

Luquet S, Lopez-Soriano J, Holst D, Fredenrich A, Melki J, Rassoulzadegan M, Grimaldi PA. 2003. Peroxisome proliferator-activated receptor δ controls muscle development and oxydative capability. *FASEB J* 17:2299-2301.

McClelland G, Zwingelstein G, Taylor CR, Weber JM. 1994. Increased capacity for circulatory fatty acid transport in a highly aerobic mammal. *Am J Physiol Regul Integr Comp Physiol* 266:R1280-1286.

McClelland GB, Kraft CS, Michaud D, Russell JC, Mueller CR, Moyes CD. 2004. Leptin and the control of respiratory gene expression in muscle. *Biochim Biophys Acta, Mol Cell Biol Lipids* 1688:86-93.

McClelland GB. 2004. Fat to the fire: The regulation of lipid oxidation with exercise and environmental stress. *Comp Biochem Physiol B: Biochem Mol Biol* 139:443-460.

McClelland GB, Brooks GA. 2002. Changes in MCT 1, MCT 4, and LDH expression are tissue specific in rats after long-term hypobaric hypoxia. *J Appl Physiol* 92:1573-1584.

McClelland GB, Hochachka PW, Reidy SP, Weber J. 2001. High-altitude acclimation increases the triacylglycerol/fatty acid cycle at rest and during exercise. *Am J Physiol Endocrinol Metab* 281:E537-544.

McClelland GB, Hochachka PW, Weber J. 1999. Effect of high-altitude acclimation on NEFA turnover and lipid utilization during exercise in rats. *Am J Physiol Endocrinol Metab* 277:E1095-1102.

McClelland GB, Hochachka PW, Weber J. 1998. Carbohydrate utilization during exercise after high-altitude acclimation: A new perspective. *Proc Natl Acad Sci U S A* 95:10288-10293.

McGillivray DG, Garland T, Dlugosz EM, Chappell MA, Syme DA. 2009. Changes in efficiency and myosin expression in the small-muscle phenotype of mice selectively breed for high voluntary running activity. *J Exp Biol* 212:977-985.

Meek TH, Lonquich BP, Hannon RM, Garland T,Jr. 2009. Endurance capacity of mice selectively bred for high voluntary wheel running. *J Exp Biol* 212:2908-2917.

Miller WC, Bryce GR, Conlee RK. 1984. Adaptations to a high-fat diet that increase exercise endurance in male rats. *J Appl Physiol* 56:78-83.

Motojima K, Passilly P, Peters JM, Gonzalez FJ, Latruffe N. 1998. Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor α and γ activators in a tissue- and inducer-specific manner. *J Biol Chem* 273:16710-16714.

Nickerson JG, Alkhateeb H, Benton CR *et al.* 2009. Greater transport efficiencies of the membrane fatty acid transporters FAT/CD36 and FATP4 compared with FABPpm and FATP1 and differential effects on fatty acid esterification and oxidation in rat skeletal muscle. *J Biol Chem* 284:16522-16530.

Passonneau JV, Lauderdale VR. 1974. A comparison of three methods of glycogen measurement in tissues. *Anal Biochem* 60:405-412.

Paulussen RA, Geelen MJH, Beynen AC, Veerkamp JH. 1989. Immunochemical quantitation of fatty-acid-binding proteins. I. tissue and intracellular distribution, postnatal development and influence of physiological conditions on rat heart and liver FABP. *Biochim Biophys Acta Lipid Lipid Met* 1001:201-209.

Pederson BA, Cope CR, Schroeder JM, Smith MW, Irimia JM, Thurberg BL, DePaoli-Roach AA, Roach PJ. 2005. Exercise capacity of mice genetically lacking muscle glycogen synthase. *J Biol Chem* 280:17260-17265.

Peter JB, Barnard RJ, Edgerton VR, Gillespie CA, Stempel KE. 1972. Metabolic profiles of three fiber types of skeletal muscle in guinea pigs and rabbits. *Biochemistry* 11:2627-2633.

Phinney SD, Bistrian BR, Evans WJ, Gervino E, Blackburn GL. 1983. The human metabolic response to chronic ketosis without caloric restriction: Preservation of submaximal exercise capability with reduced carbohydrate oxidation. *Metab Clin Exp* 32:769-776.

Powers SK, Lawler J, Dempsey JA, Dodd S, Landry G. 1989. Effects of incomplete pulmonary gas exchange on VO2 max. *J Appl Physiol* 66:2491-2495.

Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. 2007. Return to the fetal gene program protects the stressed heart: A strong hypothesis. *Heart Fail Rev* 12:331-343.

Randle PJ, Garland PB, Hales CN, Newsholme EA. 1963. The glucose fatty-acid cycle: Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 281:785-789.

Rennie MJ, Edwards RHT, Halliday D, Davies CTM, Matthews DE, Millward DJ. 1981. Protein metabolism during exercise. In: Waterlow JC and Stephen JML, editors. Nitrogen Metabolism in Man. London: Applied Science Publishers. p 509-523

Rezende EL, Garland T,Jr, Chappell MA, Malisch JL, Gomes FR. 2006. Maximum aerobic performance in lines of *mus* selected for high wheel-running activity: Effects of selection, oxygen availability and the mini-muscle phenotype. *J Exp Biol* 209:115-127.

Rezende EL, Gomes FR, Chappell MA, Garland Jr. T. 2009. Running behavior and its energy cost in mice selectively bred for high voluntary locomotor activity. *Physiol Biochem Zool* 82:662-679.

Rezende EL, Gomes FR, Malisch JL, Chappell MA, Garland T, Jr. 2006a. Maximal oxygen consumption in relation to subordinate traits in lines of house mice selectively bred for high voluntary wheel running. *J Appl Physiol* 101:477-485.

Rezende E, Kelly S, Gomes F, Chappell M, Garland Jr. T. 2006b. Effects of size, sex, and voluntary running speeds on costs of locomotion in lines of laboratory mice selectively bred for high wheel - running activity. *Physiol Biochem Zool* 79:83-99.

Roberts TJ, Weber JM, Hoppeler H, Weibel ER, Taylor CR. 1996. Design of the oxygen and substrate pathways II: Defining the upper limits of carbohydrate and fat oxidation. *J Exp Biol* 199:1651-1658.

Romijn JA, Coyle EF, Sidossis LS, Gastaldelli A, Horowitz JF, Endert E, Wolfe RR. 1993. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol Endocrinol Metab* 265:E380-391.

Rose AJ, Richter EA. 2005. Skeletal muscle glucose uptake during exercise: How is it regulated? *Physiology* 20:260-270.

Roy RR, Hutchison DL, Pierotti DJ, Hodgson JA, Edgerton VR. 1991. EMG patterns of rat ankle extensors and flexors during treadmill locomotion and swimming. *J Appl Physiol* 70:2522-2529.

Schroeder F, Petrescu A, Huang H *et al.* 2008. Role of fatty acid binding proteins and long chain fatty acids in modulating nuclear receptors and gene transcription. *Lipids* 43:1-17.

Seeherman HJ, Richard Taylor C, Maloiy GMO, Armstrong RB. 1981. Design of the mammalian respiratory system II: Measuring maximum aerobic capacity. *Respir Physiol* 44:11-23.

Sharma S, Taegtmeyer H, Adrogue J, Razeghi P, Sen S, Ngumbela K, Essop MF. 2004. Dynamic changes of gene expression in hypoxia-induced right ventricular hypertrophy. *Am J Physiol Heart Circ Physiol* 286:H1185-1192.

Siu PM, Donley DA, Bryner RW, Alway SE. 2004. Myogenin and oxidative enzyme gene expression levels are elevated in rat soleus muscles after endurance training. *J Appl Physiol* 97:277-285.

Spriet LL, Watt MJ. 2003. Regulatory mechanisms in the interaction between carbohydrate and lipid oxidation during exercise. *Acta Physiol Scand* 178:443-452.

Stanley WC, Connett RJ. 1991. Regulation of muscle carbohydrate metabolism during exercise. *FASEB J* 5:2155-2159.

Suarez RK, Darveau CA. 2005. Multi-level regulation and metabolic scaling. *J Exp Biol* 208:1627-1634.

Suzuki Y, Lanner C, Kim J *et al.* 2001. Insulin control of glycogen metabolism in knockout mice lacking the muscle-specific protein phosphatase PP1G/RGL. *Mol Cell Biol* 21:2683-2694.

Swallow JG, Koteja P, Carter PA, Garland T. 1999. Artificial selection for increased wheel-running activity in house mice results in decreased body mass at maturity. *J Exp Biol* 202:2513-2520.

Swallow JG, Carter PA, Garland T. 1998a. Artificial selection for increased wheel-running behavior in house mice. *Behav Genet* 28:227-237.

Swallow JG, Garland Jr. T, Carter PA, Zhan W, Sieck GC. 1998b. Effects of voluntary activity and genetic selection on aerobic capacity in house mice (*mus domesticus*). *J Appl Physiol* 84:69-76.

Swallow JG, Rhodes JS, Garland T, Jr. 2005. Phenotypic and evolutionary plasticity of organ masses in response to voluntary exercise in house mice. *Integr Comp Biol* 45:426-437.

Syme DA, Evashuk K, Grintuch B, Rezende EL, Garland T, Jr. 2005. Contractile abilities of normal and "mini" triceps surae muscles from mice (*mus domesticus*) selectively bred for high voluntary wheel running. *J Appl Physiol* 99:1308-1316.

Tan N, Shaw NS, Vinckenbosch N, Liu P, Yasmin R, Desvergne B, Wahli W, Noy N. 2002. Selective cooperation between fatty acid binding proteins and peroxisome proliferator-activated receptors in regulating transcription. *Mol Cell Biol* 22:5114-5127.

Tarnopolsky LJ, MacDougall JD, Atkinson SA, Tarnopolsky MA, Sutton JR. 1990. Gender differences in substrate for endurance exercise. *J Appl Physiol* 68:302-308.

Taylor CR, Weibel ER. 1981. Design of the mammalian respiratory system. I. problem and strategy. *Respir Physiol* 44:1-10.

Taylor CR. 1987. Structural and functional limits to oxidative metabolism: Insights from scaling. *Ann Rev Physiol* 49:135-146.

Teboul L, Febbraio M, Gaillard D, Amri EZ, Silverstein R, Grimaldi PA. 2001. Structural and functional characterization of the mouse fatty acid translocase promoter: Activation during adipose differentiation. *Biochem J* 360:305-312.

Templeman NM, Beaudry JL, Le Moine CMR, McClelland GB. 2010. Chronic hypoxia- and cold-induced changes in cardiac enzyme and gene expression in CD-1 mice. *Biochim Biophys Acta, Gen Subj* 1800:1248-1255.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E. 1999. Housekeeping genes as internal standards: Use and limits. *J Biotechnol* 75:291-295.

van Nieuwenhoven FA, Verstijnen CPHJ, Abumrad NA, Willemsen PHM, van Eys GJJM, van der Vusse GJ, Glatz JFC. 1995. Putative membrane fatty acid translocase and cytoplasmic fatty-acid-binding protein are co-expressed in rat heart and skeletal muscles. *Biochem Biophys Res Commun* 207:747-752.

Vega RB, Huss JM, Kelly DP. 2000. The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* 20:1868-1876.

Venables MC, Achten J, Jeukendrup AE. 2005. Determinants of fat oxidation during exercise in healthy men and women: A cross-sectional study. *J Appl Physiol* 98:160-167.

Vock R, Hoppeler H, Claassen H, Wu DX, Billeter R, Weber JM, Taylor CR, Weibel ER. 1996a. Design of the oxygen and substrate pathways VI: Structural basis of intracellular substrate supply to mitochondria in muscle cells. *J Exp Biol* 199:1689-1697.

Vock R, Weibel ER, Hoppeler H, Ordway G, Weber JM, Taylor CR. 1996b. Design of the oxygen and substrate pathways V: Structural basis of vascular substrate supply to muscle cells. *J Exp Biol* 199:1675-1688.

Vork MM, Glatz JFC, Surtel DAM, Knubben HJM, van der Vusse GJ. 1991. A sandwich enzyme linked immuno-sorbent assay for the determination of rat heart fatty acid-binding protein using the streptavidin-biotin system. Application to tissue and effluent samples from normoxic rat heart perfusion. *Biochim Biophys Acta, Gen Subj* 1075:199-205.

Wagenmakers AJM. 1998. Muscle amino acid metabolism at rest and during exercise: Role in human physiology and metabolism. *Exerc Sport Sci Rev* 26:287-314.

Wagner PD. 1996. Determinants of maximal oxygen transport and utilization. *Annu Rev Physiol* 58:21-50.

Wang Y, Zhang C, Yu RT, Cho HK, Nelson MC, Bayuga-Ocampo C, Ham J, Kang H, Evans RM. 2004. Regulation of muscle fiber type and running endurance by PPARδ. *PLoS Biol* 2:1532-1539.

Weber JM, Brichon G, Zwingelstein G, McClelland G, Saucedo C, Weibel ER, Taylor CR. 1996a. Design of the oxygen and substrate pathways IV: Partitioning energy provision from fatty acids. *J Exp Biol* 199:1667-1674.

Weber JM, Roberts TJ, Vock R, Weibel ER, Taylor CR. 1996b. Design of the oxygen and substrate pathways III: Partitioning energy provision from carbohydrates. *J Exp Biol* 199:1659-1666.

Weber J, Haman F. 2004. Oxidative fuel selection: Adjusting mix and flux to stay alive. *Int Congr* 1275:22-31.

Weibel ER, Taylor CR, Hoppeler H. 1991. The concept of symmorphosis: A testable hypothesis of structure-function relationship. *Proc Natl Acad Sci U S A* 88:10357-10361.

Weibel ER, Taylor CR, Weber JM, Vock R, Roberts TJ, Hoppeler H. 1996. Design of the oxygen and substrate pathways VII: Different structural limits for oxygen and substrate supply to muscle mitochondria. *J Exp Biol* 199:1699-1709.

Weibel ER, Bacigalupe LD, Schmitt B, Hoppeler H. 2004. Allometric scaling of maximal metabolic rate in mammals: Muscle aerobic capacity as determinant factor. *Respir Physiol Neurobiol* 140:115-132.

Welch KC,Jr, Altshuler DL, Suarez RK. 2007. Oxygen consumption rates in hovering hummingbirds reflect substrate-dependent differences in P/O ratios: Carbohydrate as a `premium fuel'. *J Exp Biol* 210:2146-2153.

Withers PC. 1977. Measurement of VO2, VCO2, and evaporative water loss with a flow-through mask. *J Appl Physiol* 42:120-123.

Wong LE, Garland T, Jr., Rowan S, Hepple RT. 2009. Anatomic capillarization is elevated in medial gastrocnemius muscle of mighty mini mice. *J Appl Physiol* 106:1660-1667.

Zhang B, Marcus SL, Sajjadi FG, Alvares K, Reddy JK, Subramani S, Rachubinski RA, Capone JP. 1992. Identification of a peroxisome proliferator-responsive element upstream of the gene encoding rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase. *Proc Nat Acad Sci U S A* 89:7541-7545.