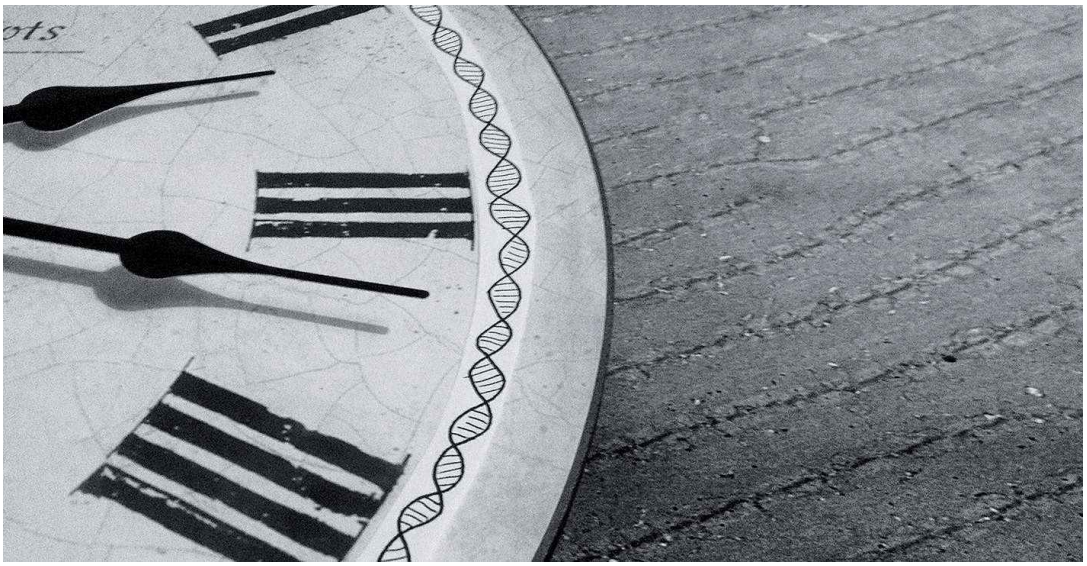
STUDIES IN SPORT, PHYSICAL EDUCATION AND HEALTH
239

Sira Karvinen

Lifespan and Skeletal Muscle Properties

The Effects of Genetic Background, Physical Activity and Aging



JYVÄSKYLÄN YLIOPISTO

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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2016

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The good thing about science is that it's true
whether or not you believe in it

-Neil deGrasse Tyson-

ABSTRACT

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Obesity and metabolic disorders have become a notable world-wide epidemic. The pathogenesis of metabolic diseases, such as metabolic syndrome and type 2 diabetes, has begun to negatively affect life expectancy of current generations. Low aerobic capacity has shown to be a strong predictor of mortality both in rodents and humans. Exercise is known to increase an individual's aerobic capacity; interestingly, recent studies have suggested that genetic background may play a significant role in the physical activity level of an individual. The purpose of this study was to investigate the role of genetic background and physical activity on skeletal muscle properties, metabolism and lifespan. The study consisted of three parts: (1) a cross-sectional voluntary running intervention in high-capacity runner (HCR) and low-capacity runner (LCR) rats, (2) a longitudinal voluntary running intervention in HCR and LCR rats, and (3) a long-term follow-up study with physical activity discordant human twins. Our study showed that low intrinsic aerobic capacity is associated with fast muscular fatigue and slow metabolic recovery after maximal muscle contractions. At the whole-body level, low intrinsic aerobic capacity was linked to low body temperature, which may play a role in the onset of gaining extra weight and, thus, developing metabolic disorders. High intrinsic aerobic capacity in turn was associated with elevated SIRT3 protein level in skeletal muscle, which is possibly linked to increased lifespan. Nevertheless, vigorous physical activity commenced at adult age did not reduce mortality or increase lifespan in rodents. High long-term participation in vigorous leisure-time physical activity did predict significantly reduced mortality in dizygotic twins; however, there was no difference in the lifespan of monozygotic twins that are genetically identical. HCRs were more physically active both in control and voluntary running groups when compared to corresponding LCR groups. Also, the persistent discordances in participation of vigorous physical activity were significantly more common in dizygotic twin pairs than in monozygotic pairs stating that genes have an influence on the persistent voluntary participation in vigorous leisure-time physical activity. Our results indicated that genetic predisposition plays a significant role in exercise participation, hence, genetic pleiotropy may partly explain the associations observed previously between high physical activity and mortality.

Keywords: aerobic capacity, genetic background, lifespan, physical activity, skeletal muscle

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hobbies and everyday life with you. With the courage and curiosity we share for life I am sure that we have a great journey ahead. Finally, I thank our adorable, cheerful twins Tiikka and Viia for their sharp-eyed, unexpected questions and joy they give me every day. I hope you never lose your curiosity to the world around you and have the courage to follow your dreams.

Jyväskylä 29.3.2016
Sira Karvinen

LIST OF ORIGINAL ARTICLES

This thesis is based on the following four original research articles, which will be referred to in the text by their Roman numerals:

I **Torvinen, S.,** Silvennoinen, M., Piitulainen, H., Närväinen, J., Tuunanen, P., Gröhn, O., L., M., Koch, L.G., Britton, S.L. & Kainulainen, H. 2012. Rats bred for low aerobic capacity become promptly fatigued and have slow metabolic recovery after stimulated, maximal muscle contractions. PloS One 7 (11), e48345.

II **Karvinen, S**., Waller, K., Silvennoinen, M., Koch, L.G., Britton, S.L., Kaprio, J., Kainulainen, H. & Kujala, U.M. 2015. Physical activity in adulthood: genes and mortality. Scientific Reports. 15;5:18259.

III **Karvinen, S**., Silvennoinen, M., Vainio, P., Sistonen, L., M., Koch, L.G., Britton, S.L. & Kainulainen, H. 2016. Effects of intrinsic aerobic capacity, aging and voluntary running on skeletal muscle sirtuins and heat shock proteins. Experimental Gerontology 79: 46–54.

IV **Karvinen, S.,** Silvennoinen, M., Ma, H., Törmäkangas, T., Ranta lainen, T., Rinnankoski-Tuikka, R., Lensu, S., Koch, L.G., Britton, S.L., & Kainulainen, H. 2016. Voluntary running aids to maintain high thermogenesis in rats bred for high aerobic capacity. *Submitted for publication.*

ABBREVIATIONS

ATP	Adenosine triphosphate
CI	Confidence interval
Cyt c	Cytochrome c
DZ	Dizygotic
HCR	High-capacity runner
HOMA-IR	Homeostasis model assessment of insulin resistance
HR	Hazard ratio
HSF	Heat-shock factor
Hsp	Heat-shock protein
LCR	Low-capacity runner
MET	Metabolic equivalent of task
MRS	Magnetic resonance spectroscopy
MZ	Monozygotic
NIH	National Institutes of Health
OXPHOS	Oxidative phosphorylation
PCr	Phosphocreatine
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
P_i	Inorganic phosphate
ROS	Reactive oxygen species
SIRT	Sirtuin
UCP	Uncoupling protein
VO_{2max}	Maximal oxygen uptake (maximal aerobic capacity)

CONTENTS

1 INTRODUCTION

Physical activity and whole-body aerobic capacity have a major role in health and longevity. However, current environment favors sedentary lifestyle that together with excessive energy intake are the main causes of overweight and metabolic diseases that have increased worldwide. Due to obesity, the current generation of children in America may have shorter life expectancies than their parents (Olshansky et al. 2005). Interestingly, despite the obesogenic environment some individuals remain resistant to weight gain. How do these obesity-resistant individuals remain lean and healthy in the current environment?

Previous studies have shown that high cardiorespiratory fitness and aerobic capacity are good predictors of reduced mortality (Myers et al. 2002; Kodama et al. 2009). In rodents, it has been shown that good intrinsic aerobic capacity, even without particular physical training, is associated with long lifespan (Koch, Britton & Wisloff 2012). Good aerobic capacity also promotes a so-called healthy phenotype, whereas low aerobic capacity is associated with obesity and increased risk of metabolic and cardiovascular diseases (Kivelä et al. 2010; Wisloff et al. 2005). Since exercise increases individuals' aerobic capacity, it is of general interest to study the effects of physical activity on longevity. Former studies have shown that physically active humans are heathier and may have longer lifespan compared to their sedentary counterparts (Löllgen, Bockenhoff & Knapp 2009; Andersen et al. 2002).

However, physical activity level varies substantially between individuals. Some people are eager to be physically active whereas others prefer sedentary lifestyle. Recent studies have suggested that there is a genetic component underlying willingness in exercise participation (Kujala et al. 2003; Stubbe et al. 2006). Indeed, animal models have shown a clear importance of genetic background on physical activity level (Novak et al. 2010; Kelly et al. 2010). Since physical activity is highly recommended to people despite their genetic background or physical status, it is important to study the effects of voluntary training in metabolism, skeletal muscle properties and lifespan with individuals who differ in their genetic background.

The present study investigated the effects of genetic background, aging and physical activity level on skeletal muscle properties, metabolism and lifespan. The study consisted of three parts: (1) a cross-sectional voluntary running intervention in HCR/LCR rats, (2) a longitudinal voluntary running intervention in HCR/LCR rats, and (3) a long-term follow-up with physical activity discordant human twins. In the cross-sectional study, we first explored the effect of intrinsic aerobic capacity on skeletal muscle properties in the untrained state. Thereafter, rats were divided into control (no running wheel) and runner groups (running wheel) and samples were collected after a one-year intervention. In the longitudinal intervention, we studied the effect of voluntary running on physical activity level and lifespan. In the human study, both dizygotic (DZ) and monozygotic (MZ) same sex twin pairs that were discordant from their physical activity level were included. In the twin study we followed the mortality of the individuals for 23 years.

The purpose of the present experiments was to determine the individual effects of genetic background, aging and physical activity on skeletal muscle properties and lifespan. Since there is some evidence of how genetics and physical activity alone affect lifespan, we combined these two factors within the same study to increase current understanding about the interplay between genes and environment.

2 LITERATURE REVIEW

2.1 Aerobic capacity

At the whole-body level, an individual's aerobic capacity refers to the maximal amount of physiologic work that an individual can perform as measured by oxygen consumption (VO_{2max}). Aerobic capacity depends on body size and composition, and is affected by the following factors: age, sex, weight, genetic background and physical activity level. In humans, the highest aerobic capacity is normally reached between 15 to 30 years, after which it gradually decreases. Men have generally higher aerobic capacity compared to women, due to sex-related differences in body composition, concentration of blood hemoglobin and heart size (Loe et al. 2013; Lee et al. 2011; Talbot, Metter & Fleg 2000; Drinkwater 1984; Cureton et al. 1986; George et al. 1995). Also, some environmental factors affect aerobic capacity; e.g. residence in high altitude increases, whereas several weeks enforced bed-rest lowers one's aerobic capacity (Frisancho et al. 1995; Wilhite et al. 2013; Capelli et al. 2006). In addition, several physiological parameters affect the maximal aerobic capacity, including heart rate, cardiac output, stroke volume and efficiency of oxygen transport at tissue level.

High aerobic capacity is strongly associated with health and longevity (Löllgen, Bockenhoff & Knapp 2009; Myers et al. 2002) as well as decreased risks of cardiovascular disease, stroke, high blood pressure and several metabolic diseases such as type 2 diabetes (Physical Activity Guidelines Advisory Committee 2008). Aerobic training can help to maintain and improve an individuals' aerobic capacity.

2.1.1 Physical activity

Endurance exercise training causes adaptations of the cardiorespiratory and neuromuscular systems that enhance the delivery of oxygen from blood to muscles cells, as well as increase the amount and efficiency of muscle mito-

chondria (Jones & Carter 2000; Baar et al. 2002; Pilegaard, Saltin & Neufer 2003). These adaptations effect an improvement in individuals' aerobic capacity and endurance performance.

The level of endurance training response depends on the duration of the exercise bouts, the intensity and frequency with which they are performed (Jones & Carter 2000; Wenger & Bell 1986). Also, individuals personal training status, sex and age affect the magnitude of the adaptations to training (Jones & Carter 2000). There is some evidence from the literature that high intensity training (80 to 100%of VO_{2max}) is the most efficient way in developing VO_{2max} both in humans and rodents (Jones & Carter 2000; Kemi et al. 2005). However, aforementioned studies show that even modest endurance training increases VO_{2max} to some extent. Furthermore, other studies have also shown a 5 to 10% improvement in VO_{2max} and increases in mitochondrial enzyme level with only a short-term endurance training program (Mier et al. 1997; Hickson 1981; Spina et al. 1996). Recently very low volume high-intensity interval training (all-out exercise efforts above 100% of peak workload) has been proposed as a novel, time-effective exercise regime for improving VO_{2max} (Saanijoki et al. 2015; Burgomaster et al. 2007).

Hickson *et al*. reported evidence that during longer-term endurance training programs, VO_{2max} will eventually stabilize (Hickson et al. 1981). According to more recent studies, it is suggested that the later improvements in endurance performance result from continued improvements in submaximal factors, such as lactate threshold and exercise economy (Jones 1998; Legaz Arrese et al. 2005).

However, not all individuals respond to physical activity similarly. There is evidence that a genetic component underlies also the response to exercise, both in humans and rodents (Hamel et al. 1986; Timmons et al. 2010; Koch, Pollott & Britton 2013). Thus, the optimal exercise volume and intensity for increasing VO_{2max} varies individually.

2.1.2 Genetics

Aerobic capacity, like several other complex physiological traits such as height and strength, is an example of a quantitative trait that demonstrates continuous variation (Koch & Britton 2001). Though the question of the heritability of aerobic capacity in humans has been addressed, there is no general agreement of how strong influence genetics have on individuals' aerobic capacity; some studies show a strong genetic component of maximal oxygen uptake (Bouchard et al. 2000; Bouchard et al. 1992), whereas other studies report only low estimates of heritability (Engstrom & Fischbein 1977; Howald 1976). In human studies, it is often challenging to resolve the relative effects of genetic background and environmental factors (e. g. endurance training) to the individual variability of aerobic capacity (Kujala et al. 1998; Perusse et al. 1989; Bray 2000).

In rodents, it is possible to selectively breed individuals that differ from their intrinsic aerobic capacity without losing the genetic variation. Koch and Britton began developing high-capacity runner (HCR) and low-capacity runner (LCR) rats from a heterogeneous starting population in 1996. After several gen-

erations, these selectively bred rat lines started to significantly differ in their maximal capability to run on a treadmill (Koch & Britton 2001). After 28 generations, the HCRs and LCRs differ already by 8.3-fold in running distance (Figure 1) (Ren et al. 2013). Furthermore, genetic background appears to play a key role in why some individuals engage in physical activity and exercise more than others, both in humans and rodents (Kujala et al. 2003; Stubbe et al. 2006; Novak et al. 2010; Kelly et al. 2010).

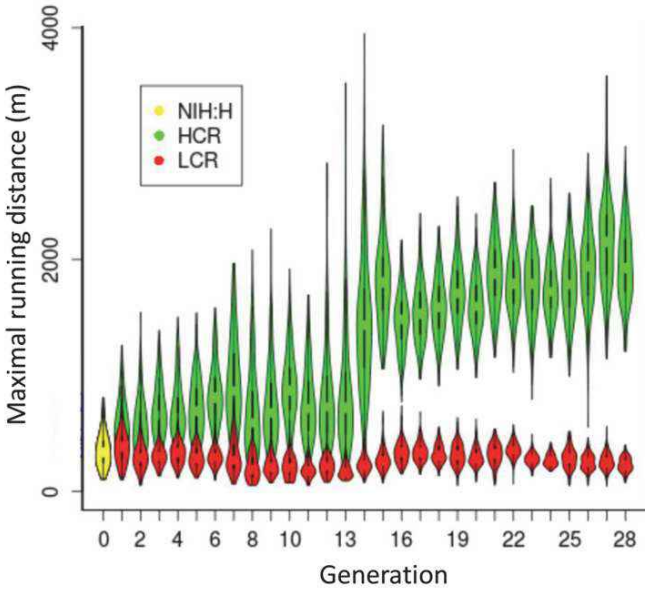


FIGURE 1 Maximal running distance of HCR/LCR rats from generations 0 to 28. Figure shows "violin-plots" for individual generations for females and males combined. The yellow oval to the left represents the founder population (NIH:H, n=153 phenotyped, out of 168), green ovals are for HCR and red LCR. Modified from Ren et al. 2013.

2.1.3 Aging

Aging is associated with a decline of maximal aerobic capacity (Buskirk & Hodgson 1987). Current evidence shows a 10 % per decade decline in VO_{2max} in both men and women (Åstrand 1960; Robinson 1938; Hawkins & Wiswell 2003). There are several potential aging-related physiological changes in the body that may contribute to the diminished aerobic capacity.

The cardiovascular system, as a vital blood and oxygen delivery system, is one key factor affecting an individual's capacity to perform physical work. The maximal heart rate has been reported to decline approximately 3-5 % per decade regardless of sex or exercise activity (Hawkins et al. 2001; Trappe et al. 1996). Also, more peripheral changes in aging contribute to the decline in aerobic capacity. Since skeletal muscles are one of the main tissues utilizing oxygen

during exercise performance, changes in body composition are evident in aerobic capacity at the whole-body level. Former studies have established a link between lean body mass and VO_{2max} in both men and women (Toth et al. 1994; Hawkins et al. 2001; Rosen et al. 1998).

The age-related decline in maximal aerobic capacity can be delayed via physical activity. Studies in aging athletes have shown lower decline rates in VO_{2max} compared to sedentary people, ranging between 5-7 % (Pollock, Miller & Wilmore 1974; Fuchi et al. 1989; Shephard 1966; Hagberg 1987).

2.1.4 Skeletal muscle

Skeletal muscle comprises about 40% of body mass and is one of the main tissues utilizing oxygen during physical activity. Skeletal muscle is also an important contributor to aerobic capacity at the whole-body level, as majority (> 80%) of cardiac output is redistributed to skeletal muscle during maximal exercise (McArdle, Katch & Katch 2015, p. 347).

In skeletal muscle, the aerobic capacity of individual muscle fibers is the maximum rate at which they can utilize oxygen. This in turn depends on muscle properties, such as capillarization and mitochondrial volume and efficiency (Holloszy 1967; Poole & Mathieu-Costello 1996). Maximal oxygen consumption (VO_{2max}), can be calculated also at the muscular level by: Q (CaO_2 - CvO_2), where Q is skeletal muscle blood flow and CaO_2 and CvO_2 are arterial and venous O_2 contents (Richardson 2000). There are four main limiting factors of VO_{2max}; (1) pulmonary diffusing capacity, (2) cardiac output, (3) blood O_2 carrying capacity and (4) skeletal muscle oxygen utilizing capacity (Bassett & Howley 2000). Recent studies have shown that O_2 delivery, and not skeletal muscle O_2 extraction, is seen as the primary limiting factor for VO_{2max} in exercising humans (Gonzalez-Alonso & Calbet 2003; Bassett & Howley 2000). Nevertheless, as being one critical factor for VO_{2max}, metabolic adaptations in skeletal muscle are essential for improving endurance performance.

Skeletal muscle properties determine the maximum rate at which muscles can utilize oxygen; however, skeletal muscle composition varies a lot between individuals. The most prominent parameters affecting skeletal muscle oxygen utilization are myosin heavy chain composition, skeletal muscle capillarization and mitochondrial volume and efficiency (Robinson et al. 1994; Pringle et al. 2003; Blomstrand, Radegran & Saltin 1997).

2.1.4.1 Muscular contraction

Muscular contraction is the activation of tension-generating sites within muscle fibers. During contraction, actin and myosin filaments slide past each other causing the length of the sarcomere to decrease.

Muscular contraction can be divided into three different stages: (1) excitation, (2) contraction and (3) relaxation (Figure 2). During excitation the action potential from the neuron spreads inside the myocyte along T-tubules causing calcium ions to be released from sarcoplasmic reticulum. Myosin heads bind to

actin filaments forming cross-bridges. During contraction, calcium ions bind to troponin and cross-bridges of myosin are attached to actin filaments generating a power stroke that moves the filaments past each other. The relaxation phase starts when calcium-ions are pumped back to the sarcoplasmic reticulum, adenosine triphosphate (ATP) then binds to myosin head and myosin cross-bridges are detached from the thin filaments.

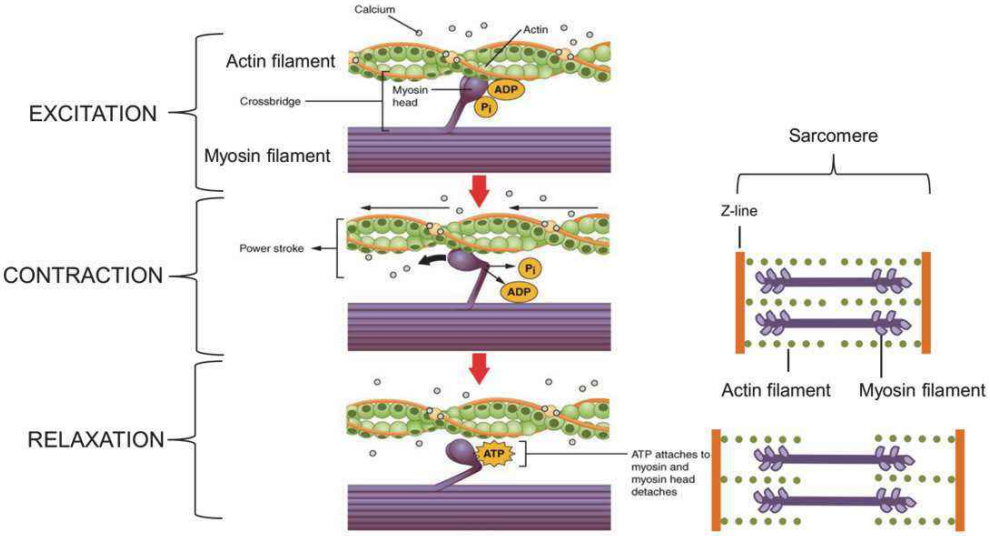


FIGURE 2 Schematic illustrations of the stages of muscle contraction. Modified from Candela Open Courses; Anatomy and physiology.

Muscular contractions can be described based on three variables: tension length and speed. According to these variables muscle contraction can be divided to three different sub-categories:

1) Isometric: muscle tension changes but the muscle-tendon complex length remains the same
2) Isotonic: muscle tension remains the same but muscle-tendon complex length changes
3) Isokinetic: muscle contraction occurs with the same speed

In spontaneous movements that underlie locomotor activity, muscular contractions involve all of the three sub-categories at different phases.

2.1.4.2 Muscular fatigue

Muscular fatigue is characterized with a decline of force production caused by prolonged activation of skeletal muscle. The capacity of muscle to resist fatigue is determined by muscle oxidative capacity, which is mainly determined by muscle mitochondrial volume and efficiency (Bassett & Howley 2000; Holloszy & Coyle 1984). Muscle fiber type composition in turn affects the oxidative ca-

pacity of a single fiber: Type I fibers are mainly oxidative and have high mitochondrial content, type IIa/x is both oxidative and glycolytic and type IIb is mainly glycolytic. (Bogdanis 2012; Carroll, Riek & Carson 2002; Burke et al. 1973; Edstrom & Kugelberg 1968).

At a cellular level, the reduced force can be caused by various metabolic or ionic changes in the myocyte, such as reduced pH, accumulation of inorganic phosphate (P_i) into myocytes, decreased intracellular calcium ion (Ca^{2+}) release or reduced sensitivity of the myofilaments to Ca^{2+}-ions (Westerblad et al. 1998; Allen et al. 1992). These changes in myocellular metabolites during muscular fatigue are often linked to each other. For example, reduced pH and accumulation of P_i lead to reduced Ca^{2+} sensitivity and reduced maximum tension. This in turn has an important contribution to the force decline, especially with repeated maximal muscle stimulation (Westerblad et al. 1991).

2.2 Energy metabolism

Energy metabolism at the whole-body level is the process of generating energy (in the form of ATP and heat) from nutrients. It comprises numerous interconnected pathways that several enzymes catalyze. The energy production pathways in cells can be divided into two categories: (1) glycolytic (anaerobic) and (2) oxidative (aerobic) energy production.

The anaerobic glycolysis takes place in the cell cytosol by an oxygen independent pathway. Compared to aerobic energy production glycolysis is fast yet produces less energy. Aerobic energy production, called oxidative phosphorylation, takes place in the mitochondrial inner membrane. During oxidative phosphorylation ATP is produced from ADP via the ATP synthase channel. This is driven by the proton motive force. Oxidative phosphorylation is also called 'coupling' since it connects electron and proton transfer to phosphorylation (Figure 3).

2.2.1 Thermogenesis

Thermogenesis is the process of heat production in organisms. It has two major functions: (1) thermoregulatory thermogenesis; that regulates the body temperature in warm blooded animals, and (2) metaboregulatory thermogenesis; during which an organism decreases its' metabolic efficiency by dissipating extra energy as heat rather than storing it as triglycerides.

There are three thermogenic methods to produce the heat: (1) exercise-associated thermogenesis, (2) non-exercise activity thermogenesis and (3) diet-induced thermogenesis. In exercise-associated thermogenesis muscular movement causes the increase in temperature whereas non-exercise activity thermogenesis mainly occurs in brown adipose tissue via uncoupling proteins (UCP). In diet-induced thermogenesis energy expenditure is increased shortly after having a meal due to nutrient processing.

Body temperature is tightly associated with metabolic rate (Landsberg 2012; Geiser 1988; Heikens et al. 2011); in fact one degree C rise in temperature is associated with a 10–13% increment in oxygen consumption (Landsberg 2012). The elevation in temperature itself is responsible for speeding up metabolism, since many enzyme-catalyzed reactions are enhanced in higher temperatures (Landsberg et al. 2009). Hence, the efficiency of an individual to produce heat may be a key factor in the control of energy metabolism at the whole-body level.

At the cellular level, the efficiency of mitochondria and UCPs play the main role in thermogenesis (Rousset et al. 2004; Cannon & Nedergaard 2004). UCP1 is primarily found in the mitochondria of brown adipose tissue, whereas UCP3 is the main uncoupling protein expressed in skeletal muscle. These proteins generate heat by non-shivering thermogenesis, which is called uncoupling at the cellular level. In uncoupling the protons bypass the ATP synthase channel and leak inside mitochondrial matrix via UCPs (Figure 3). Thus, instead of loading ATP from ADP, energy is dissipated as heat. Besides UCPs, also the amount and efficiency of mitochondria to produce heat affects body temperature.

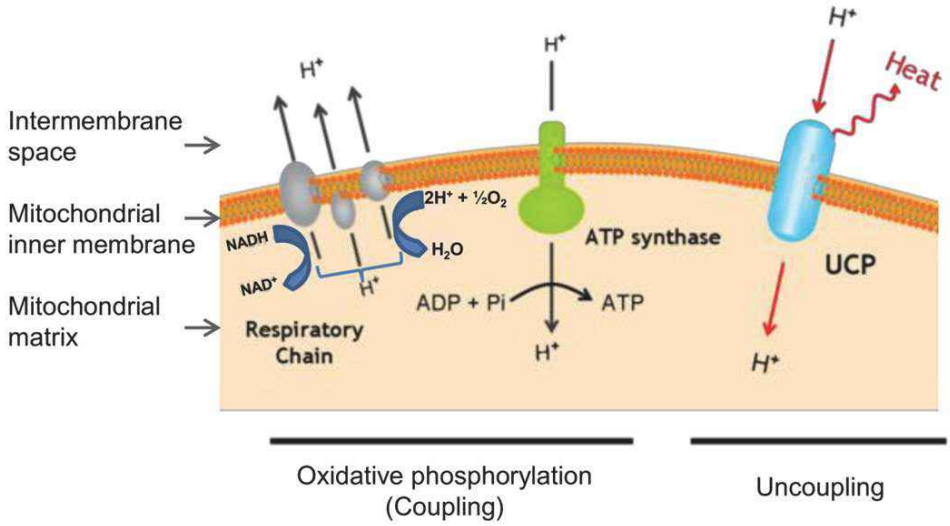


FIGURE 3 Schematic illustration of oxidative phosphorylation (coupling) and uncoupling in the mitochondria. Modified from a figure designed by Jaume Fords Martos.

Brown adipose tissue has been considered the main thermogenic organ for a long time (van den Berg et al. 2011). Nowadays, in addition to brown adipose tissue, skeletal muscle has been suggested to significantly contribute to thermogenesis (Gavini et al. 2014). The contribution of skeletal muscle mitochondrial uncoupling to weight gain has been demonstrated in several mouse models (Son et al. 2004; Costford et al. 2008; Choi et al. 2007).

2.3 Aging and lifespan

Aging is associated with an overall loss of function at the level of the whole organism that has origins in cellular deterioration. It can be influenced by many factors that can be classified as intrinsic (e.g. genetic factors) or extrinsic (e.g. environmental factors). Since aging phenotypes at the whole-body level results from a complex set of changes at the cellular level, several theories have been formulated to address the underlying mechanisms behind this phenomenon. Many of these theories share similarities and overlap with each other to some extent.

Genetic theory of aging states that an individual's genome determines lifespan. Validity of this theory is obvious in certain severe genetic diseases such as progeria and Down's syndrome (Saigal & Bhargava 2012; Alexander et al. 2015). Recent qualitative and quantitative genetic analysis of longevity has revealed that extended lifespan is frequently associated with enhanced metabolic capacity and response to stress (Jazwinski 2000). The autoimmune theory of aging declares that with age, the immune system tends to lose efficiency and experiences severe dysfunction, evidenced by autoimmunity, and a decreased ability to respond to immune challenges, such as infections (Prelog 2006). Innate immunity has also been linked to the pathogenesis of age-related chronic degenerative diseases, which have an effect on lifespan (Licastro et al. 2005). Neuroendocrine theory suggests that changes in the central nervous system precede the changes in metabolism and cognitive function (Fabris 1991). The theory of somatic mutations sees the mutations in mitochondrial DNA as the root of negative changes related to aging. It has been shown that increasing age in mammals correlates with accumulation of somatic mitochondrial DNA mutations and decline in respiratory chain function, which promotes the aging process (Trifunovic & Larsson 2008) .

Nowadays accumulation of oxidative stress and telomere shortening are two of the most prominent candidates of aging theories (Wei & Lee 2002; Van Remmen & Richardson 2001; Kruk, Rampino & Bohr 1995; Olovnikov 1996). The oxidative stress theory (e.g. free radical hypothesis) of aging proposes that deleterious actions of oxygen-derived radicals are responsible for the functional deterioration associated with aging. Since mitochondria are responsible for oxidation of various fuel molecules at the final stage of aerobic metabolism in the animal and human cells, they are also the major producers of reactive oxygen species (ROS) via incomplete reduction of O_2 by the electrons escaped from the electron transport chain (Wei & Lee 2002). Under normal physiological conditions, cells cope with the oxidative stress elicited by aerobic metabolism by an antioxidant defense system (Fridovich 1997; Leibovitz & Siegel 1980). However, the function of this antioxidant defense system declines with aging leading to accumulation of ROS and mitochondrial DNA damage (Sohal & Dubey 1994; Pansarasa et al. 1999).

The importance of chromosome ends i.e. telomeres in aging was first recognized in 1938 by Muller and soon after by McClintock (Muller 1938; McClintock 1941). Nowadays it is known that the ends of chromosomes consist of a repeated sequence of bases whose length decreases in every cell division. Telomeres are necessary for cell replication and critical for chromosomal stability. It has been shown that telomeres are shorter in fibroblasts from an old donor compared to a young donor (Kruk, Rampino & Bohr 1995). Also, Kruk *et al*. showed in the same study that the telomeric DNA repair efficiency is lower in cells from an old donor compared to cells from a young donor. The decline in the telomeric repair system seen with aging is proposed to have a functional significance to the age-related decline in genomic stability.

2.3.1 Genetics

Aging as a complex process might involve thousands of genes as well as non-genetic factors. Like many physiological traits related to aging, such as aerobic capacity and skeletal muscle properties that are to some extent genetically coordinated, there are also genes and gene sets affecting individuals' lifespan. Findings from animal studies have provided evidence that even individual genes can have a significant effect on lifespan.

Many mutations that have been shown to extend lifespan in invertebrates affect stress-response genes or nutrient sensors. In yeast cells, SIRT1 ortholog Sir2 was shown to promote longevity, which raised the interest of the role of sirtuin proteins in mammalian aging (Kaeberlein, McVey & Guarente 1999). Sirtuins interact with telomeric chromatin as well as several components of the DNA repair machinery making them potential targets for study of the mechanisms of oxidative stress on aging (Nakagawa & Guarente 2011; Michishita et al. 2008). Sirtuins also act as energy sensor proteins that respond strongly to caloric restriction; a well-recognized intervention to increase lifespan of lower organisms as well as rodents (Bishop & Guarente 2007; Rogina & Helfand 2004).

In humans, the clustering of late deaths in families with many extremely long-living individuals has provided support for a genetic component to longevity (Gudmundsson et al. 2000; Schoenmaker et al. 2006). In twin studies, it has been estimated that genetic differences account for about a quarter of the variance in adult human lifespan (Herskind et al. 1996; Skytthe et al. 2003). As in invertebrates and rodents, certain genetic variants have been shown to associate to longevity in humans, such as SIRT3, apolipoprotein E and mitochondrial genome variants (Corder et al. 1996; Rose et al. 2003; De Benedictis et al. 1999).

In rodents, high intrinsic aerobic capacity is also strongly associated to health and longevity (Löllgen, Bockenhoff & Knapp 2009; Myers et al. 2002; Koch, Britton & Wisloff 2012). However, since aerobic capacity is a polygenic trait, it is not known which genes or combinations of genes are behind the long lifespan.

2.3.2 Skeletal muscle

In humans, mortality rate and pathogenesis of several age-related diseases is associated with functional capacity and mass of skeletal muscle (Metter et al. 2002; Nair 2005). Epidemiological studies in humans also suggest that skeletal muscle aging is a risk factor for the development of several age-related diseases such as metabolic syndrome, Alzheimer's disease and cancer (Demontis et al. 2013). These findings suggest that muscle tissue may be one key regulator of systemic aging.

Aging is associated with progressive decline of organ and tissue function and accumulation of oxidative damage and DNA mutations (Wei & Lee 2002; Van Remmen & Richardson 2001). Both in humans and rodents, DNA mutations are also highly prominent in the muscle of aged individuals (Wang et al. 2001; Szczesny, Tann & Mitra 2010). Furthermore, the accumulation of dysfunctional mitochondrial proteins during aging is higher, and the levels of the antioxidant enzymes are lower in mouse skeletal muscle compared to other tissues (Szczesny, Tann & Mitra 2010). These findings raise the possibility that the muscle acts as an aging-sensitive tissue and the earlier onset of age-related degeneration in muscle may affect aging at the whole-body level (Demontis et al. 2013). Aging in general is associated with a reduction in mitochondrial function and capacity (Lopez-Lluch et al. 2008; Anderson & Prolla 2009), which eventually can be seen as a loss of physical function at the whole-body level.

Since aerobic capacity and muscle functional capacity are also important predictors of mortality in humans (Myers et al. 2002; Ruiz et al. 2011; Metter et al. 2002), endurance exercise is a potential way to rescue aging-related mitochondrial defects and, therefore, promote health and prolong lifespan. Previous studies have also shown that exercise can potentially extend lifespan both in humans and rodents (Ruiz et al. 2011; Holloszy 1993).

2.3.3 Physical activity

Observational follow-up studies report a beneficial association of physical activity with all-cause mortality as compared to inactivity mortality in humans (Löllgen, Bockenhoff & Knapp 2009; Blair, Cheng & Holder 2001; Williams 2001). Meta-analyses further demonstrate a dose-response curve especially from sedentary subjects to those with low and moderate exercise intensity (Löllgen, Bockenhoff & Knapp 2009; Samitz, Egger & Zwahlen 2011). Löllgen *et al* highlighted a non-linear risk-reduction curve with an increasing amount of physical activity (Löllgen, Bockenhoff & Knapp 2009) (Figure 4).

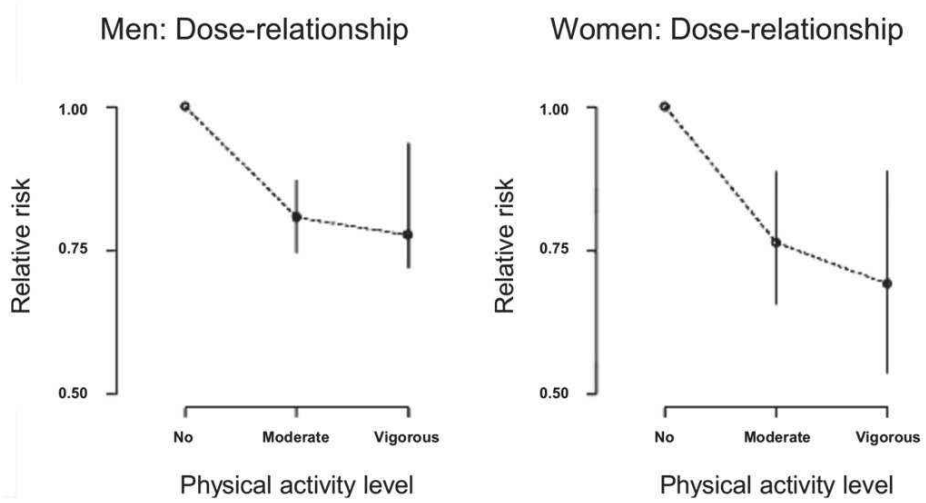


FIGURE 4 Physical activity dose-response relationship to mortality. Data is based on meta analytical results of multivariate-adjusted estimates for men and women in studies with three levels of activities (estimates plus 95 % confidence intervals). Modified from Löllgen et al. 2009.

It seems that there may be a saturation curve when presenting the association between physical activity level and mortality. A recent study even suggested a U-shaped association between all-cause mortality and dose of jogging (Schnohr et al. 2015). In Schnohr's study, light and moderate joggers had lower mortality than sedentary subjects, whereas strenuous joggers had similar mortality rate as the sedentary group. According to these studies it may be sufficient to exercise moderately to achieve a healthy lifestyle; moderate physical activity seems to reduce all-cause mortality effectively in both human and rodents without increasing risks due to possible cardiovascular side-effects of high intensity exercise (Baggish & Wood 2011; Navarro et al. 2004).

In rodents, an early study on the effects of exercise on the lifespan of rats showed that voluntary running shortened the survival of rats (Slonaker & J. R. 1912). However, non-pathogen free rats were used in this study, and chronic infections may have affected the outcomes. Later, Benedict *et al.* similarly found that exercise shortens the lifespan of rats (Benedict & Sherman 1937). In this study rats started the exercise during middle-age or during old age, varying from about 12 to 26 months of age at the beginning of intervention. Starting the exercise intervention at this late age may affect the outcomes of the study; indeed, there is some evidence that the age of onset of regular exercise plays a significant role in the life expectancy in rodents. In a study by Edington *et al.*, exercise increased the longevity of male rats if the exercise was begun at an early age (prior to 400 days) (Edington, Cosmas & McCafferty 1972) (Figure 5). However, in this study the exercise was not voluntary wheel running but a relatively low intensity exercise on a treadmill. Nevertheless, similarly as in rodents, it seems that physical activity does not improve longevity in humans if com-

menced in adulthood (Edington, Cosmas & McCafferty 1972; Goodrick et al. 1983; Kujala, Kaprio & Koskenvuo 2002).

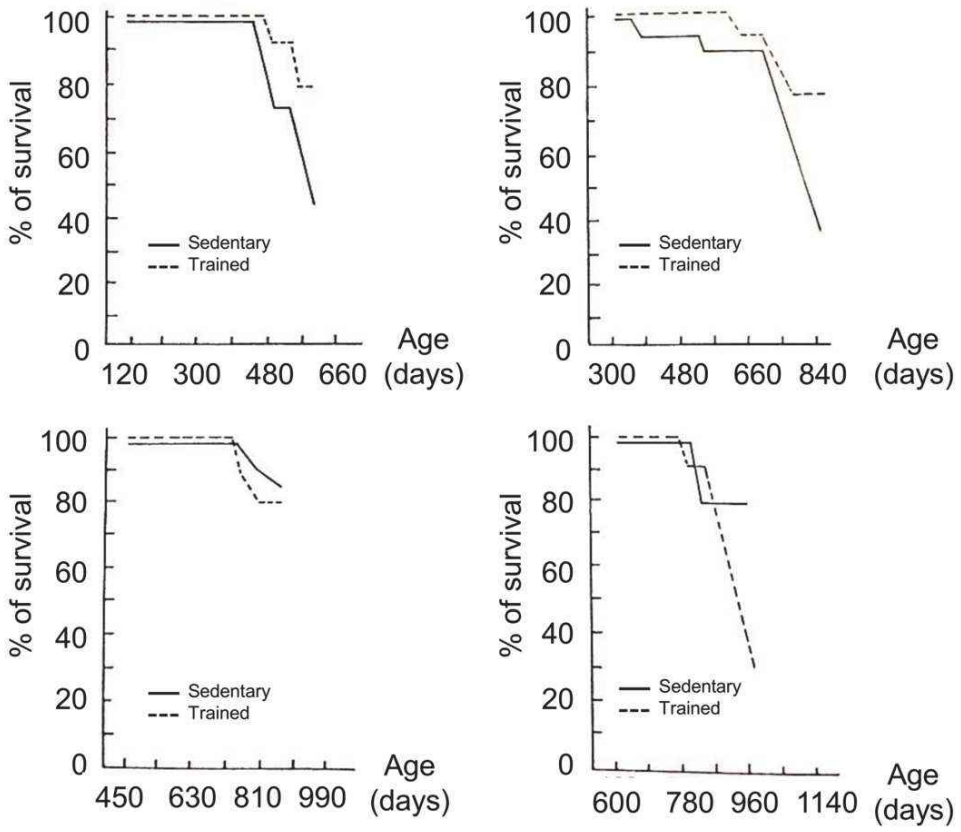


FIGURE 5 Evidence of a possible threshold age on the benefits of exercise for longevity. Figures represent the relative survival of groups that started the training intervention at different ages. Modified from Edington et al. 1972.

More recently, two studies on specific-pathogen-free male Long-Evans and Wistar rats confirmed that exercise results in improved survival but does not improve maximal lifespan (Holloszy et al. 1985; Goodrick 1980). In Goodrick's study the rats started the intervention at an age of 1.5 months and in Holloszy's at the age of 9 months. However, because male rats do not compensate the increased energy expenditure by increasing food intake, it is not clear whether their improvement in average longevity was due to decreased availability of energy (e.g. caloric restriction) or due to exercise. In general, female rats tend to increase their food intake in response to increased energy expenditure (Holloszy 1993; Titchenal 1988), which makes them better suited for studying the effects of exercise without the possible interference of caloric restriction. In Holloszy's study, when female rats were given running wheels at the age of 5 months,

runners had a significant prolongation of average longevity without an increase in maximal lifespan (Holloszy 1993) (Figure 6).

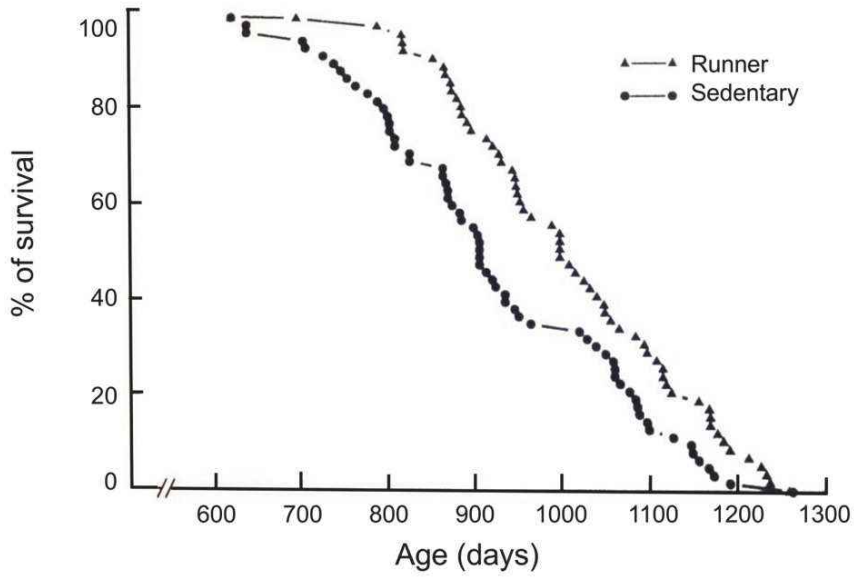


FIGURE 6 Effect of voluntary wheel running compared to sedentary on longevity of female Long-Evans rats. Modified from Holloszy et al. 1993.

Since male rats that have an access to running wheels do not have an increase in maximal longevity despite a relative caloric deficit, the possible effect of exercise-induced oxidative stress seemed a possible mechanism to prevent a maximum lifespan-extending effect of a caloric restriction related to voluntary running. Holloszy *et al.* conducted a study to determine if antioxidants would allow a maximal longevity-extending effect of exercise to manifest itself in male rats. However, the antioxidant diet had no effect on longevity of the runners, stating that the caloric deficit induced by exercise in male rats does not have a life-extending effect that is countered by oxidative tissue damage (Holloszy 1998). In mice, life-long spontaneous exercise was found not to prolong lifespan but to improve health span (Garcia-Valles et al. 2013). In this study voluntary running did prevented several aging-related signs of frailty (such as decreases in strength, endurance and motor coordination), which was accompanied by a significant increase in mitochondrial biogenesis in skeletal muscle.

High cardiorespiratory fitness is known to be a strong predictor of reduced mortality, even more robust than physical activity level itself when these two are measured within the same study (Lee et al. 2011). Indeed, studies investigating reliability of physical activity questionnaire show that they are less valid than a quantitative assessment of physical capacity or fitness by exercise testing (Jacobs et al. 1993; Lee et al. 2011). In general, physical activity is effective for disease preventive purposes. However, the magnitude of effects with simi-

lar levels of training intensity and frequency differ in individual subjects due to genetic factors (Timmons et al. 2010; Koch, Pollott & Britton 2013) as well as the age when regular training was initiated.

2.4 Study populations

A critical point when beginning a research project is to select a proper cell line, animal model or human population for the particular study question to be investigated. The HCR/LCR rat model system is widely used to study aerobic capacity and complex metabolic diseases. Twin cohorts are commonly used in human studies where the effects of genetic variation need to be controlled.

2.4.1 HCR/LCR rat model system

HCR/LCR rats are contrasting rat lines that differ from their inborn (genetic) aerobic capacity. Koch and Britton began developing HCR and LCR rats from a heterogeneous starting population of 168 rats in 1996 (Koch & Britton 2001). Selection for low and high capacity was based upon distance run to exhaustion on a treadmill using a speed-ramped running protocol; the initial speed was 10 m/min and it was increased by 1 m/min every 2 min (slope constantly at 15 degrees). From the starting population the best runners were chosen to breed the HCR line and the rats that ran the shortest distance were used as founders for the LCR line. At each generation, within-family selection was practiced using 13 families for rotational breeding for both the low and high capacity lines.

The original founder population ran to exhaustion in 355 ± 11 m on average. After six generations of selection the rat lines already differed in running capacity by 171%, with most of the change occurring in the HCR line (Koch & Britton 2001). After 18 generations of breeding the low and high capacity lines differed in maximal running capacity by 615 % (Kivelä et al. 2010). VO_{2max} was 12 % higher in HCRs after 7 generations and 24 % higher after 22 generations of selection compared to LCRs (Gonzalez et al. 2006; Johnsen et al. 2013). Whereas VO_{2max} is a direct measure of aerobic capacity, maximal running capacity may be influenced by factors other than VO_{2max}, such as anaerobic capacity, resistance to fatigue, efficiency and oxygen cost of running and motivational factors leading to larger difference in running capacity between the rat lines that could be expected solely based on VO_{2max} results (Koch et al. 2011).

The advantage of the HCR/LCR rat model compared to animal models produced by mutations is that HCR/LCR rat lines are not inbred but maintained as genetically heterogeneous as possible. Compared to inbred strains, in which almost all allelic variation has been taken to fixation, outbred selected lines maintain genetic complexity (Koch et al. 2011). Genetic variation allows combinations of allelic variants at multiple interacting loci to be enriched in response to selection pressure (Carlborg et al. 2006).

HCR/LCR rats have been extensively studied. At the whole-body level, in addition to poor running capacity, LCRs have lower physical activity level, higher body weight gain and increased complex disease risk compared to HCRs (Kivelä et al. 2010). Furthermore, LCRs have increased cardiovascular disease risk and higher susceptibility to tachyarrhythmia's than HCRs (Wisloff et al. 2005). Previous studies have also shown that LCRs have poor capillarization, less subsarcolemmal and intermyofibrillar mitochondria, and reduced mitochondrial respiratory capacity in skeletal muscle compared to HCRs (Kivelä et al. 2010; Rivas et al. 2011; Walsh et al. 2006). In liver tissue, LCRs exhibit reduced hepatic mitochondrial oxidative capacity that in turn increases susceptibility to hepatic steatosis and liver injury (Thyfault et al. 2009). In response to a high-fat diet, LCRs also have increased hepatic lipid accumulation compared to HCRs (Morris et al. 2011).

On the other hand, compared to LCRs, HCRs represent a lean, healthy phenotype with decreased metabolic risk factors, higher physical activity level and increased energy expenditure (Kivelä et al. 2010; Novak et al. 2010). The difference in fuel selection has been associated to the healthy phenotype of HCRs, which more efficiently oxidizes fatty acids and branched-chain amino acids than LCRs (Overmyer et al. 2015). HCRs also seem to be protected against certain negative metabolic changes related to environmental and hormonal factors, such as high-fat diet and ovariectomy (Vieira-Potter et al. 2015; Noland et al. 2007; Morris et al. 2014).

The first studies of survivability revealed that lifespan segregated strongly with running capacity between the lines (Figure 7). The median age of death was 30.1 months for HCR rats and 23.5 months for LCR, representing a 28% difference in lifespan (Koch et al. 2011). There was no significant difference in maximal lifespan between females and males within lines. Standard necropsy profiles demonstrated that age-related lesions were not different in incidence or severity between LCR and HCR rats (Koch et al. 2011).

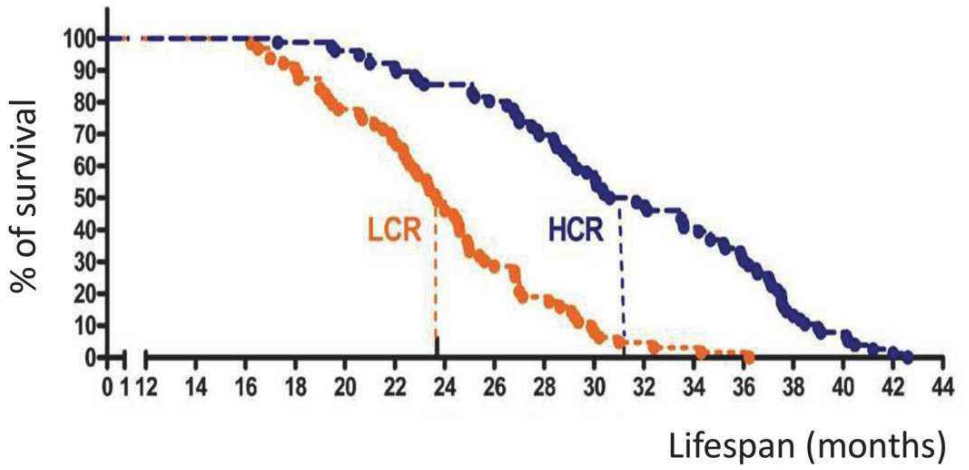


FIGURE 7 Survival curves for data combined from HCR/LCR rats from generations 14, 15, and 17 (nLCR = 63; nHCR = 76). Data includes both female and male rats that lived in standard rat cages. Modified from Koch et al. 2011.

2.4.2 Twin studies

Using twin studies it is possible estimate the contribution of genetics to a given trait or condition of interest. The classic twin study design relies on studying twins raised in the same family; the sharing of genetic similarity (MZ pairs) or dissimilarity (DZ pairs) and intrauterine and subsequent environments make twins unique subjects for genetic studies (Tan et al. 2015). Depending on the study question either monozygotic (MZ) or dizygotic (DZ) twins are used or a combination of the two. MZ (identical) twins derive from a single fertilized egg and are therefore genetically identical. DZ twins share about 50 percent of their segregating genes, the same as non-twin siblings.

The classical assumptions in twin study design are: (1) random mating, (2) equal environments, (3) minimal gene-environment interaction and (4) representativeness of the general population. Random mating assumption states that that people are as likely to choose partners who are different from themselves as they are to choose partners who are similar for a particular trait. If this assumption failed, DZ twins could share either a greater or lower percentage of their genes than expected. In twins research it is also assumed that twins raised in the same family experience the same environment. Classical twin design hypothesizes that genes and environment have only separate and distinct contributions to certain trait. However, also interactions between genes and environment appear to influence the development and intensity of traits. Finally, twin studies in general assume that twins are no different from the general population in terms of the trait. When these assumptions made in the classical twin design are met, it is a powerful tool for partitioning genetic from environmental factors on a studied trait. (Rijsdijk & Sham 2002).

In the classical twin study design, the observed resemblance in MZ twins versus DZ twins allows the disintegration of the phenotypic variation into additive genetic (A), common or shared environmental (C), and unique environmental or residual effects (E) (Figure 8). Additive genetic influences result from the sum of allelic effects across multiple genes; in MZ pairs the correlation for allelic effects is 1 and in DZ pairs 0.5. The environmental effects shared by the twins consist of an intrauterine environment, home environment and family socioeconomic status. The unique environmental factors are unshared with the twin pair, such as personal lifestyle and experiences, or stochastic biological effects. The variance of each twin is due to their genetics, shared environmental effects and residual factors that are unique to each twin, i.e. A+C+E. (Tan et al. 2015).

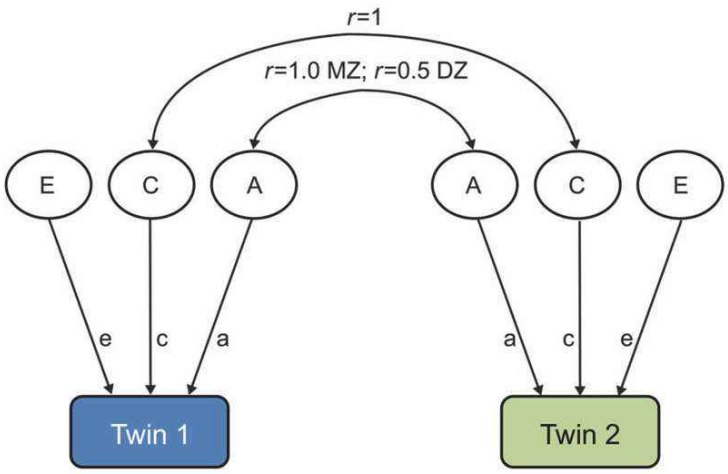


FIGURE 8 Path diagram for the classical twin study design. The figure shows the additive genetic influences (A), common or shared environmental effects (C) and unique environmental or residual effects (E) between a MZ and DZ twin pair (r=correlation). Modified from Tan et al. 2015.

In research, particularly discordant twin pairs offer a valuable setup for studies. Discordance in twin studies means that a clear difference exists between a twin pair in a certain trait, such as smoking status or physical activity level. The information from discordant twins is used in a design that is referred to as the co-twin control method. The value of this design is that one of the twins serves as control for the other which enables to distinguish between associations that reflect causality and associations that result from the confounding effects of genes or environmental factors (van Dongen et al. 2012). A unique advantage of the MZ twin design is the ability to study biological discordance against a genetically equivalent background.

Twin studies are a valuable source of information about the genetic basis of complex traits. Generally, twins can be used to obtain insights into the genetic epidemiology of complex traits and diseases, to study the interaction of geno-

type with sex, age and lifestyle factors, and to study the causes of co-morbidity between traits and diseases. There are several national twin registers worldwide, which can offer unique opportunities for selected sampling for quantitative trait loci linkage and association studies (Boomsma, Busjahn & Peltonen 2002).

3 PURPOSE OF THE STUDY

The purpose of this thesis was to study the effects of genetic background, physical activity and aging on skeletal muscle properties and lifespan. Since it was already shown in rodents that intrinsic aerobic capacity is a major determinant of longevity, we further investigated how genetic aerobic capacity combined with acquired aerobic capacity via voluntary running affects lifespan. Additionally, we utilized a human twin cohort to study how genetic background and physical activity affect lifespan in a human population. Former human studies have suggested an association between high physical activity level and long lifespan, yet the genetic background of the subjects has not been controlled in these studies. With the HCR/LCR rat model we included both cross-sectional and longitudinal intervention study setups. With human twins we utilized a long-term observational follow-up study.

The specific aims were to study:

1. The effect of intrinsic aerobic capacity on skeletal muscle metabolism and contractile properties during maximal electrical stimulation (I).

   Hypothesis: Low intrinsic aerobic capacity is associated with fatigue sensitivity and poor intramuscular pH homeostasis during maximal electrical stimulation, followed by slow metabolic recovery.

2. The effects of intrinsic aerobic capacity, voluntary running and aging on body temperature and heat generation during glucose tolerance test (IV).

   Hypothesis: HCR rats have intrinsically higher body temperature compared to LCRs due to their higher mitochondrial capacity to produce heat. Aging decreases body temperature levels due to ag-

ing-related loss of mitochondrial function and decreased expression of the uncoupling proteins in skeletal muscle and brown adipose tissue, but the difference between the HCR/LCR rat lines is still evident. Voluntary running increases body temperature in both rat lines due to increased mitochondrial biogenesis, thus aiding prevention of age-related decrease in mitochondrial function. Glucose injection increases body temperature, especially in HCRs, since brown adipose tissue as an insulin sensitive tissue may have a major role in dissipating extra energy as heat.

3. The effects of intrinsic aerobic capacity, voluntary running and aging on skeletal muscle SIRT, heat-shock factor (HSF) and heat-shock protein (Hsp) contents (III).

   Hypothesis: HCRs have higher expression of Hsps, SIRT and HSF proteins compared to LCRs due to their verified longer lifespan and proposed better capability to compensate aging-related oxidative stress. The response of voluntary running to the studied protein levels is higher in HCRs, since HCRs are known to engage in more voluntary activity on a running wheel and to be spontaneously more active than LCRs.

4. The effect of genetic background and adulthood physical activity on mortality (II).

   Hypothesis: Physical activity increases the mean lifespan both in rodents and human twins. Depending on the genetic background, some individuals are voluntarily more active than others, thus the most active ones have the longest increase in mean lifespan. HCRs that have an access to running wheel have longer lifespan than corresponding controls - similarly LCRs in the running group have longer lifespan compared to the controls, though this difference in lifespan is expected to be smaller than in HCRs due to the less active behavior of LCRs.

4 MATERIALS AND METHODS

4.1 Animal model

The HCR/LCR contrasting rat model was produced by two-way artificial selection, starting from a founder population of 168 genetically heterogeneous rats (N:NIH stock) (Koch & Britton 2001). N:NIH was derived from 8 inbred strains that were outcrossed into making N:NIH stock. Briefly, endurance running capacity was assessed on a treadmill and the total distance ran during the test was used as a measure for intrinsic aerobic capacity. From the founder population of 168 rats 153 were phenotyped. Rats with the highest running capacity from each generation were bred to produce the HCR line, and rats with the lowest capacity were bred to produce the LCR line.

For the study protocol described here, 169 female rats (84 HCR and 85 LCR) were obtained from generations 23-27. Each rat was phenotyped for maximal treadmill running capacity at the University of Michigan (Ann Arbor, Michigan, USA) with a speed-ramped treadmill running test (15° slope, initial velocity of 10 m × min^{-1}, increased 1 m × min^{-1} every 2 min), when rats were ~ 3 months of age. Rats were housed in an environmentally controlled facility (12/12 h light-dark cycle, 22°C) and received water and standard feed *ad libitum*.

4.1.1 Ethics statement

Experimental procedures with the rats conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU) and were approved by the National Animal Experiment Board, Finland (Permit number ESAVI-2010-07989/Ym-23).

4.1.2 Intervention (II-IV)

Until the age of 9 months, rats were housed in standard cages, 2/cage. After collecting the first treadmill running and glucose tolerance tests each group (HCR and LCR) was divided into two sub-groups matched for body weight and maximal running capacity; two sub-groups were placed in standard cages (controls: HCR-C and LCR-C) and two sub-groups were placed in cages with running wheels (voluntary running groups: HCR-R and LCR-R)(Figure 9). During the voluntary running intervention, rats in all four groups were housed 1/cage. Voluntary running was followed through a computerized system.

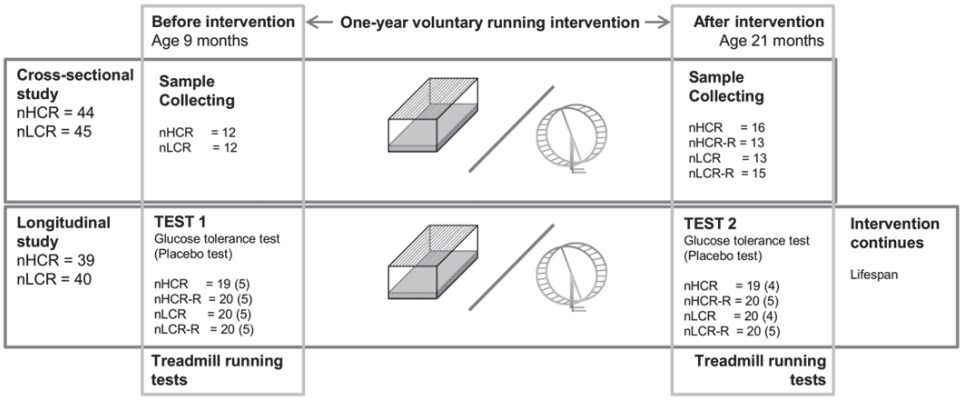


FIGURE 9 Schematic representation of the study protocol. The present study consisted of cross-sectional and longitudinal study setups with a one-year intervention of 4 sub-groups: HCR (control), HCR-R (runner), LCR (control) and LCR-R (runner). The n of each group is marked in the figure; in tests 1 and 2 the first number states the n during the glucose tolerance test and the number in parentheses describes the n during the placebo test.

4.1.2.1 Cross-sectional study (I, III, IV)

The cross-sectional study provided information of the HCR/LCR rats at the tissue level before voluntary training (untrained stage, study I) and after one year of voluntary running intervention (Figure 9, studies III and IV). There were two time points in the study: when the rats were aged 9 and 21 months. Right before these time points the maximal treadmill running capacity of the rats was tested by similar speed-ramped treadmill running tests as described above. The rats were sacrificed at these time points and tissue and blood samples were collected.

4.1.2.2 Longitudinal study (II)

Information of the lifespan of HCR/LCR rats was collected from the longitudinal study. Before these time points, maximal treadmill running capacity of the rats was tested at 9 and 21 months of age. A glucose tolerance test was per-

formed in all of the rats at the same time points and to one sub-group of rats also as a placebo test (Figure 9).

Voluntary running was followed through a computerized system throughout the lifespan. Follow-up began on the day of randomization and ended at death. Animals were euthanized when any one of the predetermined humane end-point criteria were observed.

4.1.2.2.1 End-point criteria (II)

Humane end-point criteria were utilized in the longitudinal study. The criteria for euthanasia were: movement disabilities, difficulty to maintain an upright position, assuming a crouched position for >48 h, laborious breathing, dehydration, severe loss of body mass (>20% total body mass), chronic diarrhea or constipation for >48 h, test results that indicate loss of internal organ function, any prolonged leak from a body orifice, self-harming behavior, and unresponsiveness to external stimuli. When one of these end-point criteria was met, the animal was euthanized and tissue samples were collected.

4.1.3 ^{31}P-MRS measurements (I)

^{31}P-Magnetic resonance spectroscopy (^{31}P-MRS) is a method that enables measuring the concentrations of the major phosphorus compounds in a contracting muscle, *i.e.*, PCr and P_i non-invasively. With these parameters, one can estimate the energy production efficiency of the muscle, such as the speed of PCr degradation and resynthesis, provided that simultaneous force output is recorded (Giannesini et al. 2001).

The non-invasive MRS investigation setup used in our study was modified from the protocol designed by Giannesini et al. (Giannesini et al. 2005). Rats were deprived of food for 2 h before measurements. Basal body temperature and mass were measured at rest before each investigation. Rats were anesthetized in an induction chamber with 4% isoflurane mixed in 30% O_2 and 70% N_2O. The right lower hind-limb was shaved and conductive electrolyte gel was applied at the heel and knee levels to optimize electrical stimulation of the triceps surae muscle complex. Each rat was placed in a home-built cradle designed for non-invasive functional investigation of the right triceps surae. The cradle had a built-in strain-gauge sensor (HBM, 1-LY41-6/1000, Darmstadt, Germany) and two transcutaneous electrodes to elicit and measure maximal twitch responses under isometric conditions. The foot was positioned on the pedal and the lower hind-limb was centered on a flat radio frequency (RF) coil used for MRS measurement (Figure 10).

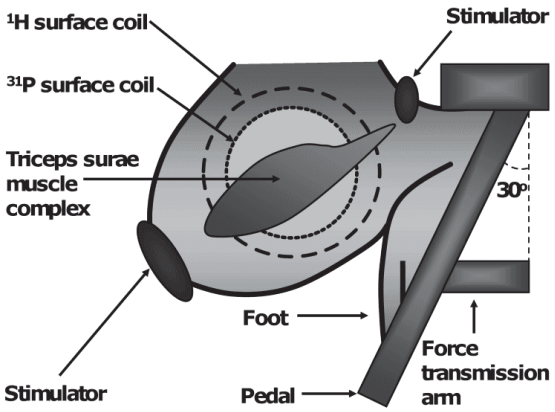


FIGURE 10 Schematic representation of the setup for measuring rat triceps surae muscle complex function with MR investigation. Triceps surae contractions were induced indirectly via electrical stimulation at the heel and knee levels. Muscle performance was measured with a force transducer which was attached to the pedal. Information about the leg position was acquired with a ^{1}H-MRS surface coil and muscle metabolic functions were studied with ^{31}P-MRS surface coil.

The pedal was held constant at a 30 degree angle. Supramaximal square-wave pulses (1-ms duration, current range: 10–19 mA, altered to maximize twitch force) were delivered transcutaneously with a constant current stimulator (Digitimer Stimulator DS7, Digitimer Ltd., Hertfordshire, U.K.) to obtain the maximal isometric twitch responses. Throughout the experiment, anesthesia was maintained by a gas inhalation through a facemask continuously supplied with 1.2–1.75% isoflurane in 30% O_2 and 70% N_2O. Corneas were protected from drying by application of ophthalmic cream (Viscotears, Novartis Pharmaceuticals, U.K.). The facemask was connected to open-circuit gas anesthesia equipment. During anesthesia, animal body temperature was maintained with a heated pad.

4.1.3.1 Electrical muscle stimulation (I)

The stimulation protocol consisted of baseline (8 min), stimulation (6 min) and recovery (8 min) measurements. Repeated isometric muscle twitches were elicited through electrical stimulation to the triceps surae muscle complex at a frequency of 3.33 Hz (in total 1200 stimuli). Force signals from the strain-gauge sensor were amplified, converted to digital signals by a 32-bit analog-to-digital converter (Power 1401, CED Ltd., Cambridge, U.K.), and processed using dedicated software (Signal software, CED Ltd.).

4.1.3.1.1 Signal Analysis (I)

From each individual twitch response, maximal force was analyzed throughout the 6 min stimulation period. Thereafter, twitch properties were averaged over 15 second epochs (in total 24 averages of 50 stimuli). In addition, twitch force was normalized with their maximum value during the stimulation to compute relative rate of reduction (% of maximum sec^{-1}) during the time-course of transcutaneous electrical stimulation. A linear trend line was adjusted over 100 twitch responses in individual time-intervals that showed rapid linear reduction in the analyzed variables.

4.1.4 Treadmill running tests

Treadmill running tests were performed first at the University of Michigan (at the age of 3 months) and similarly at the University of Jyväskylä (at the age of 9 and 21 months). Rats were tested with a speed-ramped treadmill running test (15° slope, initial velocity of 10 m × min^{-1}, increased 1 m × min^{-1} every 2 min). The maximum speed of the treadmill at the University of Jyväskylä was 60 m/min, limiting the maximal endurance performance to be 1470 m with the speed-ramped protocol used. If a rat reached this maximal speed of the treadmill, the test was finished and the endurance capacity was marked to be the maximum of the treadmill (1470 m).

4.1.5 Glucose tolerance and placebo tests (IV)

The glucose tolerance and placebo tests were performed twice, first at the age of 9 months and thereafter at the age of 21 months. Rats were deprived of food for 5 h before measurements. The running wheels of the runner rats were blocked 5 h before the measurements disabling the movement of the wheel to avoid the possible acute effects of running on the measurements. Body weight was measured before each test. At time point 0, either 2 g/kg of glucose (20 % solution) or equal volume of placebo (physiological saline solution) was injected into the peritoneal cavity. Blood glucose was measured from samples obtained from saphenous vein at time points F (fasting), 0, 30, 60, and 120 min after the injection. Blood samples for further analyses were collected before starting the glucose/placebo protocol (fasting sample).

4.1.5.1 Body temperature (IV)

Body temperature was measured with a rectal probe (Fluke 52 k/J Thermometer) at pre-intervention measurements, baseline measurements and during the glucose tolerance and placebo tests.

4.1.5.1.1 Pre-intervention measurement (IV)

Body temperature was assessed after 2 h of fasting from the same rats as used for the intervention before dividing the rats into separate intervention groups (n = 10/group). The measurement was repeated twice in separate days and the average of the two measurements was used in the statistical analyses.

4.1.5.1.2 Baseline measurements (IV)

Body temperature was measured as described above during baseline measurements to study the effects of line, aging and voluntary running on body temperature before and after one-year voluntary running intervention. Measurements were done after 5 h of fasting from the same rats as used in the intervention (n = 5/group). The average of the two fasting (F) time point measurements from each rat both during glucose tolerance and placebo tests were used in the statistical analyses.

4.1.5.1.3 Heat generation (IV)

Body temperature (Fluke 52 k/J Thermometer) was measured at time points 0, 30, 60, and 120 min after the injection. To compare the estimate of heat generation during placebo and glucose tolerance tests we calculated the area under curve (AUC) values of rectal temperature of each group during both tests between time points 0-120 min normalized with 0 min level. AUC values were used for the statistical analyses of the effect of strain, running and treatment on heat generation.

4.1.6 Spontaneous activity (II, IV)

4.1.6.1 Life-long follow-up (II)

Total spontaneous physical activity was measured every three months for four days throughout each rat's lifespan. For this purpose, a ground reaction force recording was used, as described previously (Silvennoinen, Rantalainen & Kainulainen 2014). From that data, the absolute values of the differences between consecutive force values were calculated as described by Silvennoinen *et al* (Silvennoinen, Rantalainen & Kainulainen 2014). The mean of the absolute values were calculated for every second from total 20 values per second. To obtain a single value for total spontaneous activity, the 1-s means were summed for the total measurement time and the sum was divided by the body mass (kg) of the measured rat. From that data, activity index was calculated the as a sum of three day activity from 8 a.m. to 8 a.m. per each month.

4.1.6.2 Activity during glucose tolerance and placebo tests (IV)

Spontaneous activity of the rats was followed throughout the glucose tolerance and placebo tests with ground reaction force recordings as described above

(4.1.5.1 Life-long follow-up). The rat cages were placed on top of individual ground reaction force plates 30 min before the start of the experiments and removed from the plate after the last blood glucose measurement. From this data an activity index was calculated, and the data used in statistical analyses were presented as 30 min averages. The total activity during the tests was calculated as a sum of the activity index from the whole test period.

4.1.7 Blood analyses (II, IV)

Blood glucose was measured in fresh samples (HemoCue Glucose 201 RT). Insulin was measured with ELISA from frozen (-80 °C) serum samples (Mercodia, Rat Insulin ELISA). HOMA-IR was calculated as the product of fasting glucose and insulin levels, divided by a constant (Cacho et al. 2008), with the following equation: (Glucose (mmol/l) * Insulin (μg/l))/2.430.

4.1.8 Tissue processing (III, IV)

Gastrocnemius muscle (n=4-10/group) samples were collected from HCR/LCR rats before and after the one-year voluntary running intervention. The snap-frozen samples were homogenized in liquid nitrogen and processed with either of the following protocols:

Protocol 1: HSFs and Hsps 27 and Hsp70 (III):
The muscle homogenates (n=4-5/group) were lysed in lysis buffer (24 mM HEPES [pH 7.4], 100 mM NaCl, 5 mM EDTA, 0.5% Triton-X-100, 200 mM β-glycerolphosphate, 20 mM PNPP, 100 μM ortovanadate), and protein concentration was measured with the Bradford method.

Protocol 2: Other studied proteins (III, IV):
The homogenized muscle sample (n=9-10/group) was dissolved in ice-cold buffer (20 mM HEPES [pH 7.4], 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM, b-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% NP-40, 0.2% sodium deoxycholate, and 3% protease and phosphatase inhibitor cocktail [P 78443; Pierce, Rockford, IL]). The muscle homogenate was then centrifuged at 10 000 *g* for 10 min at 4°C. Total protein content was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) with an automated KoneLab instrument (Thermo Scientific, Vantaa, Finland).

Protocol 1 was a standard protocol in the biochemistry laboratory in the University of Turku, whereas protocol 2 was the standard protocol in University of Jyväskylä. Hence HSFs, Hsps27 and Hsp70 that were analyzed in the University of Turku were processed with a different protocol than the rest of the studied proteins.

4.1.9 Western blot analyses (III, IV)

4.1.9.1 HSF1, HSF2 and Hsp27 (III)

Samples containing 35 µg of total protein were separated by SDS-PAGE on 7.5 % gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes with the semi-dry method. Transfer efficiency was checked with Ponceau S staining, and the membranes were boiled for 15 min before being blocked with 5% milk in PBS-0.3%-Tween 20 and immunoblotted with the following primary antibodies: α-HSF1 (1:1000; SPA-901, Enzo Life Sciences), α-HSF2 (1:200; Clone 3E2, Cayman Chemicals), α-HSP27 (1:1000; SPA-800, Enzo Life Sciences), α-GAPDH (1: 15000; ab9485, Abcam). After the primary antibody incubation, membranes were washed in PBS-T and incubated with a suitable secondary antibody. The signal was developed using Amersham ECL Plus substrate (GE Healthcare) and quantified using ChemiDoc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories). HSF and Hsp27 protein levels were normalized to the corresponding GAPDH band.

4.1.9.2 Other studied proteins (III, IV)

Aliquots of muscle homogenate were solubilized in Laemmli sample buffer and heated at 95°C to denaturate proteins (except for total OXPHOS Cocktail heated at 50°C). Samples containing 30 µg of total protein were separated by SDS-PAGE for 60 to 90 min at 200 V using 4–20% gradient gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to PVDF membranes at 300 mA constant current for 2 h on ice at 4°C. The homogeneity of protein loading was checked by staining the membrane with Ponceau S. Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% non-fat dry milk for 2 h and then incubated overnight at 4°C with commercially available polyclonal primary phosphospecific antibodies to measure the following protein contents with stated dilutions: GAPDH (1:1000; ab9485, Abcam), tubulin (1:1500; T6199, Sigma), PGC-1α (1:4000; 516557, Calbiochem), cytochrome c (1:500; sc-8385, Santa Cruz biotechnology, Inc.), UCP2 (1:300; ab67241 Abcam), Total OXPHOS Cocktail (1:1000; ab110413; Abcam), SIRT3 (1:800; ab118334). All antibodies were diluted in TBS-T containing 2.5% non-fat dry milk.

After the primary antibody incubation, membranes were washed in TBS-T, incubated with a suitable secondary antibody, diluted in TBS-T with 2.5% milk for 1 h followed by washing in TBS-T. Proteins were visualized by ECL according to the manufacturer's protocol (SuperSignal West femto maximum sensitivity substrate, Pierce Biotechnology) and quantified using ChemiDoc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories). The UCP3 and pACC membranes described above were incubated in Restore Western blot stripping buffer (Pierce Biotechnology) for 30 min and re-probed with GAPDH by immunoblot analysis as described above. Results were normalized to the corresponding level of GAPDH, tubulin or PonceauS stained

actin band. Thereafter, the mitochondrial SIRT3 was normalized to OXPHOS Cocktail level.

4.2 Human subjects (II)

The Finnish Twin Cohort retains records of all same-sex twin pairs born in Finland before 1958, if both co-twins were alive in 1967 (Kaprio & Koskenvuo 2002). In 1975, a baseline questionnaire was sent to twin pairs that were both alive at that time. A second questionnaire was sent in 1981 to all twin pairs; and a third questionnaire was sent in 1990 to all twins aged 33–60 years that had responded to at least one of the earlier questionnaires. The determination of zygosity was based on an accurate, validated questionnaire (Sarna et al. 1978).

The twin study setup included a 15-y segment, where participation in physical activity was reported at three time-points (1975, 1981 and 1990), and in total 23-year follow-up to evaluate mortality (from 1990 to 2013).

4.2.1 Ethics statement

A statement from the ethics committee of the Department of Public Health, University of Helsinki approved our study. The study was conducted according to the declaration of Helsinki. All participants gave their informed consent by providing questionnaire responses. The participants were provided regular feedback on the purpose and conduct of the study and were informed that they may withdraw from the study at any time.

4.2.2 Assessment of leisure-time physical activity level

Leisure physical activity was assessed with identical validated questions on surveys conducted in 1975 and 1981and with modified questions on the survey conducted in 1990. Assessments of vigorous physical activity were based on intensity categories applied at all three time points. The categories were: 1) walking, 2) alternately walking and jogging, 3) jogging (light running), and 4) running. Vigorous activity was defined as levels 2, 3, or 4 (more intensive than normal walking), performed at least 3-5 times per month in 1975 and 1981, or at least for 30 min each week in 1990.

To characterize the amount of long-term leisure time physical activity performed, MET index was calculated, based on a series of structured questions that covered leisure physical activity and any physical activity performed during the journey to and from work (Kujala et al. 1998, Waller, Kaprio & Kujala 2008). MET index was calculated by assigning a multiple of resting metabolic rate to each activity and by calculating intensity x duration x frequency of activity. The MET index was expressed as the sum of leisure MET-hours per day at each time-point (1975, 1981, and 1990). Lastly, mean MET index was calculated

for long-term leisure-time physical activity; i.e., the MET indexes for 1975, 1981, and 1990 were added together and divided by three.

4.2.3 Inclusion criteria

Inclusion criteria for the human twin study were: complete data on leisure-time physical activity required for calculating the metabolic equivalent of task (MET) index from three postal surveys conducted in 1975, 1981, and 1990. A total of 11 325 individuals (5113 males and 6212 females) met these criteria for all three time points, including 4190 complete twin pairs (1388 MZ pairs, 2547 DZ pairs, and 255 pairs with unknown zygosity).

Of the 4190 same-sex twin pairs 179 (4.3%) persistently discordant pairs were identified for participation in vigorous physical activity. These activity-discordant twin pairs comprised 5.3% (134 of 2547) of all DZ pairs and 2.4% (34 of 1388) of all MZ pairs ($p < 0.001$; Fisher's exact test for a difference in persistent discordances between MZ and DZ pairs). For the analysis of mortality, only persistently discordant pairs with known zygosity were included (34 MZ pairs and 134 DZ pairs).

4.3 Statistical analyses

Statistical analyses for variables were carried out using SPSS for Windows 22 statistical software (SPSS Inc., Chicago, IL, USA) or in Mplus 7. The Shapiro-Wilk test was used to investigate within-group normality for a given parameter of interest. Levene's test was conducted to assess the homogeneity of the variance assumption. When assumptions were met, independent-samples t-test was used. When the normality or equality of variance assumptions were not met, statistical comparisons of parameters between groups were made using the Mann-Whitney test. Univariate analysis was performed to analyze the line, age and running effects to the measured parameters.

Within-group comparisons between before and after intervention parameters were performed using Wilcoxon's test. Mixed-model analysis controlled for age, running (yes/no), treatment (placebo/glucose), time point (F, 0, 30, 60 and 120 min) and spontaneous activity, was used to determine the effect of line (HCR or LCR) on the measured body temperature levels. Separate analysis for both rat lines of the effect of running, treatment, time point and spontaneous activity on rectal temperature were performed using 4x5 longitudinal covariance structure analyses in Mplus 7 for both glucose tolerance and placebo tests.

In the mortality study, Mplus version 7 was used. Mortality analyses of rats were conducted in individual study groups (e.g., HCR-R vs. LCR-R); in pooled groups (HCR vs. LCR); and according to training (e.g., HCR-R vs. HCR-C). Hazard ratios (HR) and 95% confidence intervals (CI) were calculated with the Cox proportional hazard model with the StataIC13 statistical package. Fol-

low-up started on the day of randomization and ended at death. Results were adjusted for age at randomization and body weight.

In the human study, Mplus version 7 was used to estimate Intraclass Correlation Coefficients (ICC) and to partition the total variance into genetic and environmental components with standard techniques. All-cause mortality during follow-up was analyzed. The exact dates of death, causes of death, and emigration from Finland were available from the Population Register Centre of Finland. A total of 243 177 person-years were accumulated during follow-up, from the 1990 individual response date to the end of July 2013, the date of emigration, or death. First, individual-based mortality in relation to leisure-time physical activity participation was analyzed, adjusted for age and sex. HRs and 95% CIs with the Cox proportional hazard model were calculated, and clustering for family members applied. Next, the model for baseline levels of education was adjusted, smoking status, alcohol use, BMI, and work-related activity, by adding one covariate at a time to the model. These adjustments did not significantly change the HRs.

Individuals that did not participate in vigorous activity were used as the reference group in all analyses of leisure time physical activity. In addition, pairwise analyses were performed with the same models for all twin pairs. All twins (collectively) were analyzed at first, and then, MZ and DZ pairs separately. Identical analysis strategy was used for assessing work-related physical activity categories, except that data was not adjusted for work-related physical activity. Data were analyzed with the StataIC13 statistical package (Stata Corp, College Station, Texas, U.S.A.). P-values less than 0.05 were considered statistically significant.

5 RESULTS

5.1 Cross-sectional study: HCR/LCR rats

5.1.1 Intrinsic aerobic capacity and skeletal muscle properties (I)

5.1.1.1 ^{31}P-MRS (I)

^{31}P-magnetic resonance spectroscopy (MRS) results demonstrated that phosphocreatine (PCr) resynthesis after stimulation was significantly slower in LCRs when comparing the slope values of linear trend lines adjusted between 7–9 min time points ($p < 0.05$, Figure 11A). LCRs also maintained the intramuscular pH poorly compared to HCRs (Figure 11B). At the beginning of the stimulation protocol both rat lines had an equivalent pH value of 7.00. At end of the test period, HCRs had a pH of 6.78 on average, whereas LCRs had a more reduced pH of 6.55.

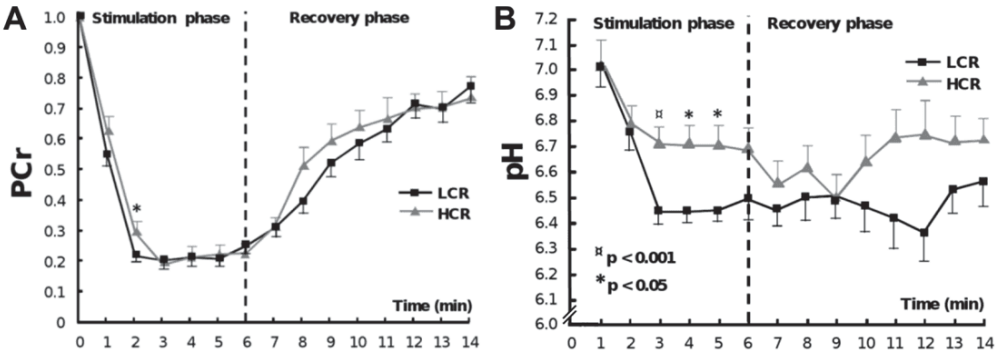


FIGURE 11 PCr and pH levels during 31P-MRS acquisition protocol (I). PCr (A) and pH (B) levels in the triceps surae muscle complex during stimulation (6 min) and recovery (8 min) measurements. PCr resynthesis was significantly slower in LCRs compared to HCRs ($p < 0.05$) when comparing the individual slope values. LCRs also had significantly lower PCr level at the 2 min time point ($p < 0.05$). Intramuscular pH was lower in LCRs throughout the protocol except for the 1 and 9 min time points. *$p < 0.050$, ¤$p < 0.001$, values are expressed as mean ± SEM.

5.1.1.2 Twitch properties (I)

LCRs tended to show higher maximal twitch force than HCRs during the first epochs (0–30 s), although this trend was not significant (Figure 12A). Later during the stimulation, LCRs showed a significantly lower twitch force from 2 min 45 s onwards compared to HCRs ($p \leq 0.05$, Figure 12A) The rate of reduction in the normalized twitch force (force % of maximum•min^{-1}) occurred more rapidly in LCRs than HCRs when the slope values of individual time-intervals were compared ($p < 0.05$, Figure 12A).

There was no significant difference in maximal twitch force at the beginning of the stimulation protocol (Figure 12B), whereas at the end of the stimulation protocol LCRs showed significantly lower maximal twitch force compared to HCRs ($p < 0.05$, Figure 12C).

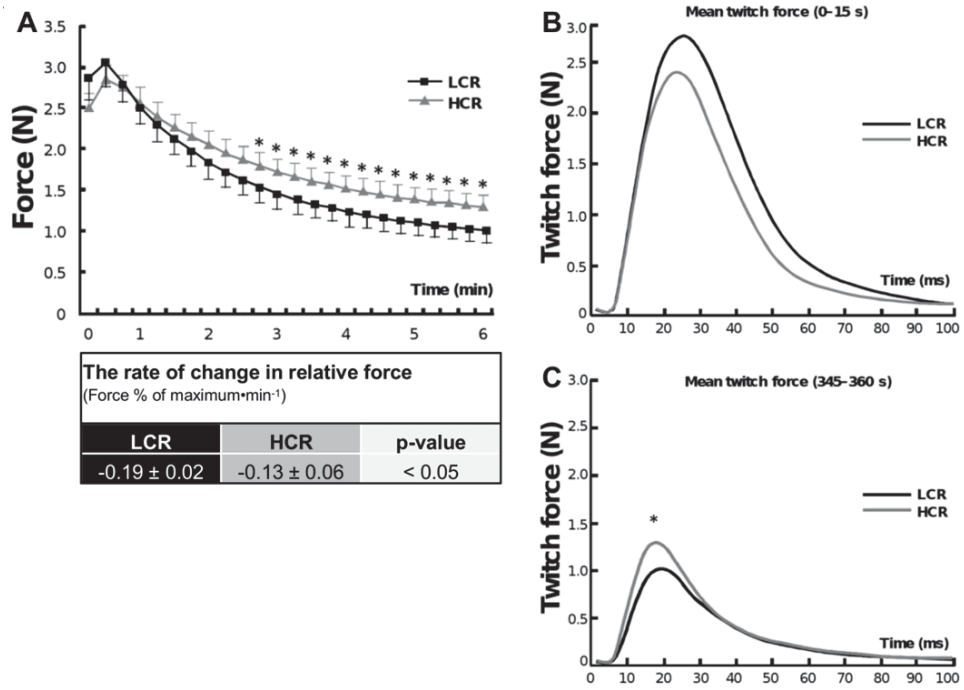


FIGURE 12 Twitch force during 6 min stimulation protocol, rate of change in relative force and initial and end values of the twitch properties (I). Force (A) measured simultaneously with MRS acquisition. From 2 min 45 s onwards LCRs had significantly lower twitch force compared to HCRs ($p \leq 0.05$). The rate of change in relative (% of maximal) twitch force was calculated in individual time-intervals (100 twitches) during the electrical stimulation (slope value). LCRs exhibited faster reduction in in normalized twitch force compared to HCRs ($p < 0.05$). The mean twitch force curves from the initial (0–15 s) (B) and last (345–360 s) epoch (C) during electrical stimulation. At the end of the stimulation protocol, LCRs showed significantly lower maximal twitch force compared to HCRs ($p < 0.05$). *$p < 0.050$, values are expressed as mean ± SEM.

5.1.2 One-year voluntary running intervention (III, IV)

5.1.2.1 Body mass and gastrocnemius muscle mass (IV)

Body mass and gastrocnemius muscle mass (relative to body mass) of the studied rat groups are listed in Table 1. Before the intervention, HCRs had lower body mass and higher relative gastrocnemius muscle mass compared to LCRs (line effect $p < 0.001$). Aging had a significant effect on both body mass and relative gastrocnemius muscle mass ($p < 0.050$); before the intervention HCRs had higher relative gastrocnemius muscle mass compared to HCRs after the intervention, while LCRs before the intervention had higher relative gastrocnemius mass compared to both LCR and LCR-R after intervention.

TABLE 1 Background information for Western blot analyses (III, IV).

Group	Body mass (g)	Gastrocnemius/Body mass (mg/g)
HCR_before	232 ± 30	5.32 ± 0.65
HCR_after	260 ± 36	4.57 ± 0.58
HCR-R_after	268 ± 34	4.78 ± 0.49
LCR_before	302 ± 26	4.73 ± 0.43
LCR_after	320 ± 37	4.21 ± 0.45
LCR-R_after	345 ± 48	4.00 ± 0.59
p-value	Line < 0.001***	Line < 0.001***
	Age < 0.050*	Age < 0.001***

Body and relative tissue mass (tissue mass/body mass) of the rats used for western blot analyses. n = 4-10/group, values are expressed as mean ± SD.

5.1.2.2 Skeletal muscle protein analysis (III, IV)

Our study showed that HCR rats had higher SIRT3, HSF1 and HSF2 content in skeletal muscle than LCRs (*gastrocnemius*, $p < 0.05$, Figure 13A, C and D). Aging significantly increased Hsp27, HSF1 and HSF2 levels ($p < 0.05$, Figure 13B-D).

Univariate analysis showed a clear line effect in PGC-1α, cyt c, UCP2 and OXPHOS protein levels ($p < 0.05$, Figure 14) with HCRs having higher protein levels compared to LCRs. Aging also increased the level of PGC-1α ($p < 0.05$) and there was a tendency for an increase in cyt c level with aging ($p = 0.087$).

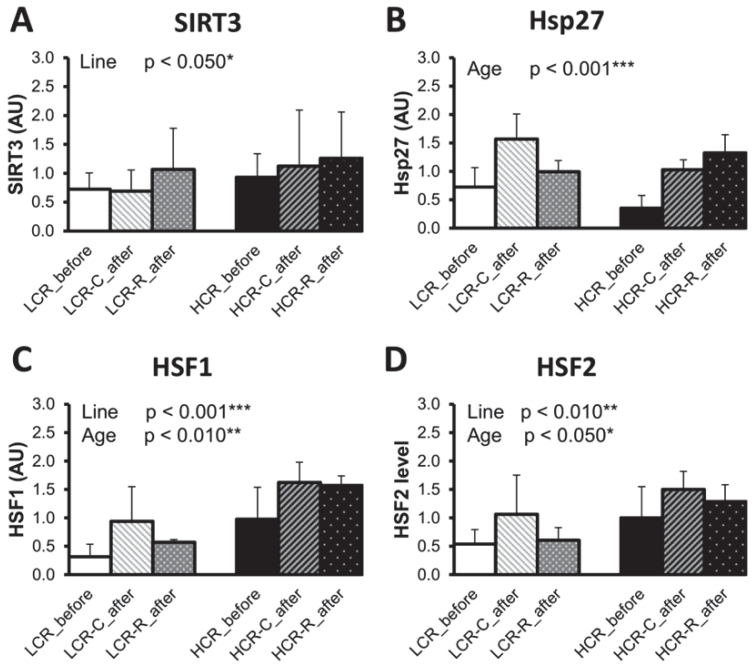


FIGURE 13 Western blot analyses from the gastrocnemius muscle (III). SIRT3 (A), n=9-10/group. Hsp27, HSF1 and HSF2 (B, C, D), n=4-5/group. Univariate analysis of the effects of rat line (HCR and LCR) and aging within both rat lines. *p < 0.050, **p < 0.010, ***p < 0.001, values are expressed as arbitrary units (AU) mean ± SD.

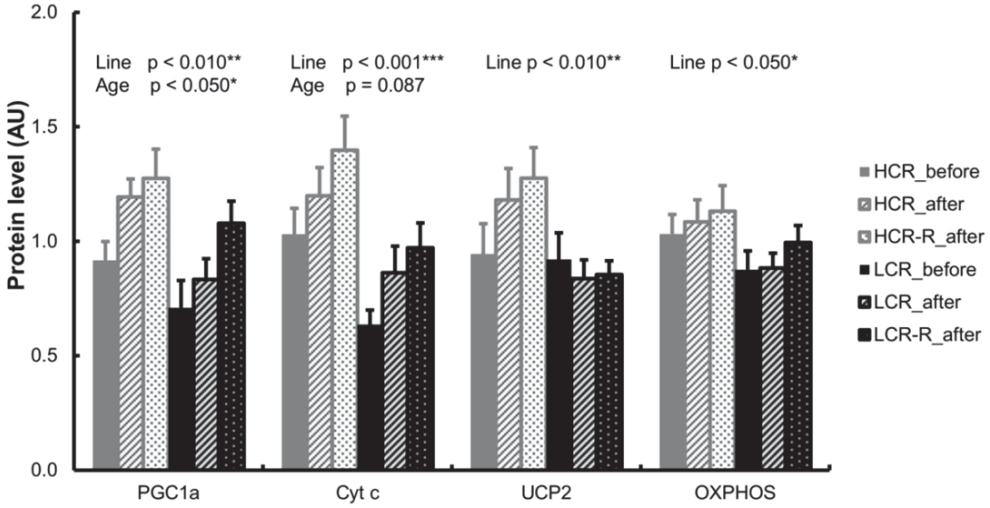


FIGURE 14 Western blot analyses from the gastrocnemius muscle (IV). n=9-10/group. Univariate analysis of the effects of rat line (HCR and LCR) and aging within both rat lines. *p < 0.050, **p < 0.010, ***p < 0.001, values are expressed as arbitrary units (AU) mean ± SEM.

5.1.2.3 Thermogenesis (IV)

The results showed that, in the untrained state, HCRs had higher body temperature compared to LCRs showing 1.3°C higher temperature level on average ($p < 0.001$, Figure 15A). Aging decreased the body temperature level of HCRs to similar levels than observed in LCRs. The opportunity to run voluntarily had a marked impact on the body temperature of HCRs ($p < 0.001$, Figure 15B) aiding them to maintain body temperature at a similar level to younger rats. Neither aging nor voluntary running had a marked impact on body temperature of LCRs.

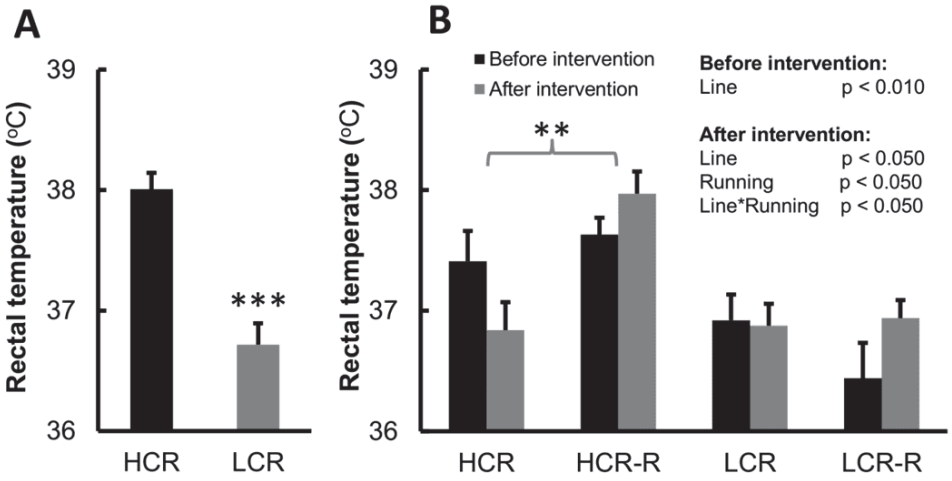


FIGURE 15 Measurements of body temperature (IV). Pre-intervention measurement of body temperature (3A). n = 10/group, ***$p < 0.001$. Baseline measurement of body temperature (3B). n = 4-5/group, **$p < 0.010$ post hoc test between HCR and HCR-R. Univariate analysis of rat line (HCR and LCR) and the effect of running before and after the one-year intervention. Values are expressed as mean ± SEM.

Glucose injection lowered the body temperature of LCRs (treatment $p < 0.050$). Compared to LCRs, HCRs were spontaneously more active (line $p < 0.001$). Before the intervention heat generation was positive during placebo and negative during the glucose test, and the LCR control group had the largest negative response to glucose injection (Figure 16A). Univariate analysis showed an effect of treatment on heat generation ($p < 0.001$). After the intervention, the treatment still had a significant effect on heat generation ($p < 0.010$, Figure 16B) and the effect of line was nearly significant ($p = 0.064$). After the intervention responses to both placebo and glucose injections in all groups were negative, with both LCR groups having a bigger negative response to glucose injection compared to HCR groups.

Univariate analysis revealed that HCRs had higher total activity during both placebo and glucose tolerance tests compared to LCRs before the one-year

voluntary running intervention (line effect $p < 0.001$, Figure. 16C). Similarly, after the one-year intervention HCRs had higher total activity during both test protocols compared to LCRs (line effect $p < 0.001$, Figure 16D).

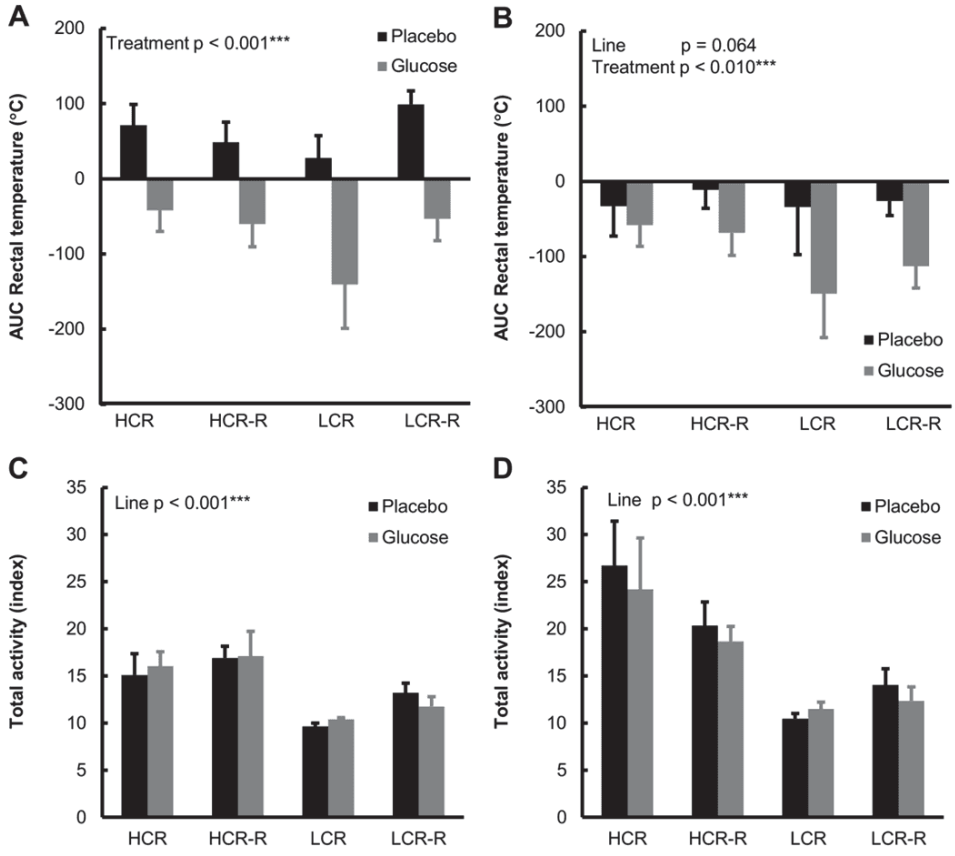


FIGURE 16 Heat generation and total activity during placebo and glucose tolerance tests before (A and C) and after (B and D) the one-year voluntary running intervention (IV). 4A and 4B: AUC Heat generation. Before the one-year intervention treatment had a significant effect on heat generation ($p < 0.001$). After the intervention treatment was still significant ($p < 0.010$) and line effect was nearly significant ($p = 0.064$). 4C and 4D: Total activity. HCRs had higher total activity compared to LCRs both before and after one-year intervention (line effect $p < 0.001$). Values are expressed as mean ± SEM.

Mixed model analyses from intervention measurements revealed, that rat line had a clear impact on body temperature both before and after the intervention ($p < 0.01$, Table 2). Longitudinal covariance analysis showed that, before the one-year voluntary running intervention, the spontaneous activity level and protocol time point (F, 0, 30, 60 or 120 min) had the largest effect on body temperature of HCRs ($p < 0.05$, Table 2), whereas in LCRs treatment (placebo/glucose injection) and spontaneous activity had highest impact on body

temperature ($p < 0.001$). After the intervention, both running and spontaneous activity had a significant contribution to the body temperature levels of HCRs ($p < 0.05$). In LCRs, treatment and time point had a marked impact on body temperature after the intervention ($p < 0.05$).

TABLE 2 Body temperature during test protocols: effect of measured parameters (IV).

	Factor	**p**
Before intervention	Line	<0.000***
HCR	Running	0.510
	Treatment	0.067
	Time point	0.036*
	Spontaneous activity	0.037*
LCR	Running	0.955
	Treatment	< 0.001***
	Time point	0.402
	Spontaneous activity	< 0.001***
After intervention	Line	<0.010**
HCR	Running	< 0.001***
	Treatment	0.815
	Time point	0.387
	Spontaneous activity	0.014*
LCR	Running	0.330
	Treatment	0.013*
	Time point	0.010*
	Spontaneous activity	0.370

Mixed-model and longitudinal covariance structure analysis of how the measured parame ters affect body temperature levels. Parameters: running (yes/no), treatment (glucose/ placebo injection), time point (F, 0, 30, 60 or 120 min) and spontaneous activity before and after the voluntary running intervention. n = 4-5 /group.

5.2 Longitudinal study: HCR/LCR rats and human twins (II)

For the longitudinal study, 79 female rats (39 HCR and 40 LCR) were obtained and testing began when the rats were ~9 months old. Figure 17A shows the study design. Rats were randomly assigned LCR and HCR rats to control (C) and running (R) sub-groups that were matched within their line for body weight (at the age of 9 months, Table 3) and endurance capacity (at the age of 3 months, Table 3): 19 HCR-C and 20 LCR-C, housed in standard cages; and voluntary runners: 20 HCR-R and 20 LCR-R, housed in cages equipped with a running wheel to permit voluntary running throughout adulthood (Figure 17A).

LCR rats weigh significantly more than HCR rats already at young age (age 3 months, $P < 0.001$, data not shown) and this difference remained signifi-

cant throughout adult life (Table 3). HCRs exhibited better endurance performance than LCRs in all of the three time points ($p < 0.001$) and rats in the runner groups had higher endurance capacity at time point 21 months compared to the corresponding control groups (HCR-C vs. HCR-R, $p < 0.001$ and LCR-C vs. LCR-R, $p < 0.005$, Table 3).

Total spontaneous physical activity was measured (horizontal and vertical movements) every three months throughout each rat's lifespan using a custom-designed force-plate system. The activity index was clearly higher among the runners than among the corresponding controls (Figure 17B). Consistent with previous reports, the HCR-C group exhibited higher spontaneous activity than the LCR-C group by 19% ($p < 0.01$, Figure 17B). In addition, for cages equipped with running wheels, the average wheel distance per day was longer in HCR-R than in the LCR-R group at time points from 12 to 29 months ($p < 0.05$, Figure 17C).

After one year of voluntary running, the LCR-R group had a significantly lower HOMA-IR index than the LCR-C group (3.5±3.0 vs. 5.7±2.8, $P < 0.05$).

TABLE 3 Best running distances (m) and body weights (g) at time points 3, 9 and 21 months. n = 16-20 / group.

Age (months)	**3**	**9**	**9**	**21**	**21**
Parameter	Best running distance (m)	Weight (g)	Best running distance (m)	Weight (g)	Best running distance (m)
Groups					
HCR-C	2142 ± 440	230 ± 23	1167 ± 262	247 ± 25	431 ± 220
HCR-R	2186 ± 355	240 ± 30	952 ± 354	286 ± 44	1295 ± 235
LCR-C	300 ± 45	290 ± 25	130 ± 48	349 ± 48	79 ± 32
LCR-R	304 ± 54	288 ± 26	155 ± 70	352 ± 49	138 ± 68
P-values					
HCR-C vs. LCR-C	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
HCR-C vs. HCR-R	n.s.	n.s.	< 0.05	< 0.01	< 0.001
LCR-C vs. LCR-R	n.s.	n.s.	n.s.	n.s.	< 0.05

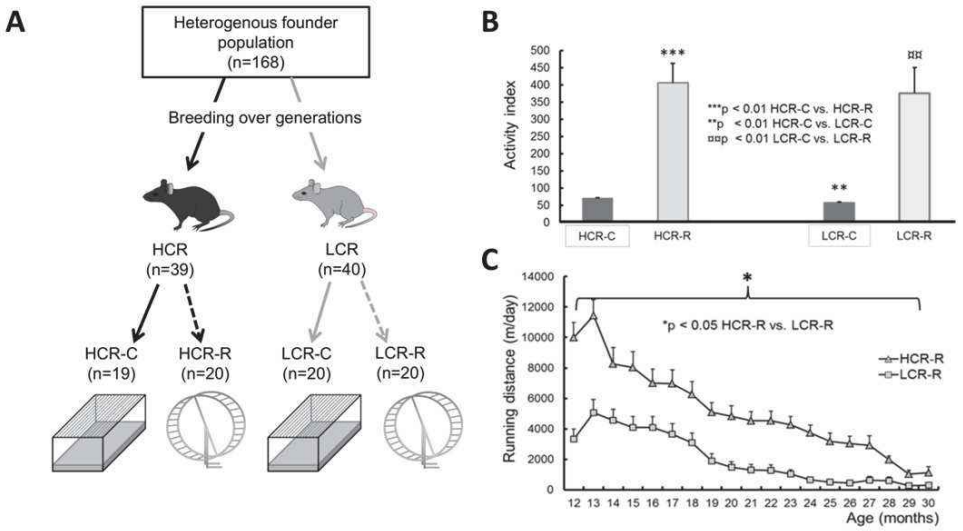
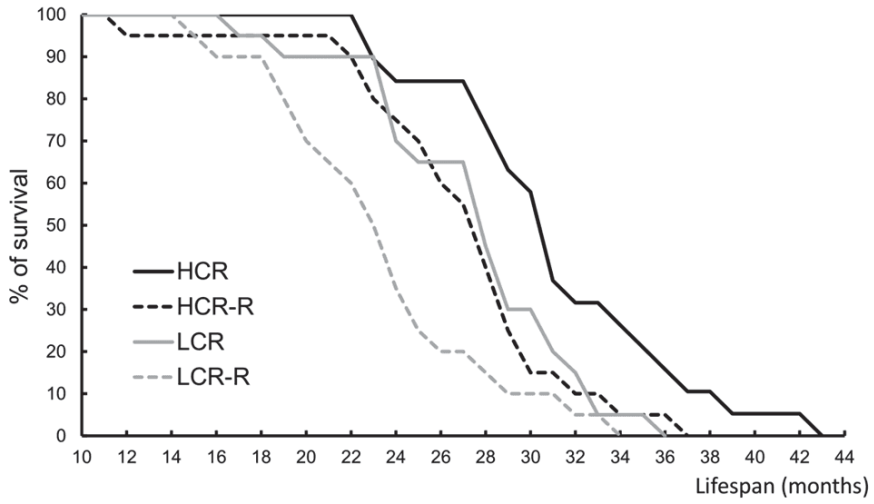


FIGURE 17 Rat study protocol and measurements (II). (A) Schematic of the study protocol. Rats were bred for high (HCR, black) or low (LCR, grey) intrinsic aerobic capacity, then assigned to control (C) or running (R) sub-groups. (B) Average daily spontaneous activity measured over 4 days between 13 and 15 months of age (activity index). Rats per group: 13 months: HCR-C=18, HCR-R=12, LCR-C=20, and LCR-R=11; 15 months: HCR-C=19, HCR-R=13, LCR-C=19, and LCR-R=10. (C) Average running distance per day. Rats per group: 12 months: HCR-R=15 and LCR-R=15; 21 months: HCR-R=16 and LCR-R=14; 30 months: HCR-R=4 and LCR-R=2. Error bars are ± SEMs.

5.2.1 Lifespan: Rat study (II)

Cox proportional hazards model showed that LCR rats had a higher risk of death than HCR rats, and the adjusted hazard ratio (HR) for HCR-R was 1.7 (95% CI: 1.1-2.6, *P* = 0.028). Surprisingly, when combined, the pool of LCR and HCR runner rats had an increased risk of death compared to control groups (HR = 2.1; 95% CI: 1.3-3.4); p < 0.001. This finding persisted after adjustments for line, age at randomization, and body weight at 9 months of age (multivariate-adjusted HR = 2.3, 95% CI: 1.4-3.6; p < 0.001).

The decreased survivability for runners vs. controls was similar for both HCR and LCR lines. The survival curves in Figure 18 shows the mean lifespan among runners was consistently 19% shorter than among controls in both the HCR (mean 26.4 vs. 31.5 months, p < 0.05) and LCR (23.8 vs. 28.4 months, p < 0.01) groups.



Lifespan	HCR-C	HCR-R	LCR-C	LCR-R	p
Mean	31.5 ± 5.3	26.4 ± 6.9	28.4 ± 5.5	23.8 ± 5.2	HCR-C vs. LCR-C < 0.05 HCR-C vs. HCR-R < 0.05 LCR-C vs. LCR-R < 0.01

FIGURE 18 Effects of genetic background and environment on lifespan (II). Control rats (C) had longer lifespans than rats in the runner groups (R) of the same line (HCR-C vs. HCR-R, P < 0.05 and LCR-C vs. LCR-R, P < 0.01). Mean lifespans were also significantly differed between rat lines (HCR-C vs. LCR-C, P < 0.05). Values in the table are means ± SDs.

5.2.2 Lifespan: Twin study (II)

The activity-discordant DZ pairs showed a difference in mortality (HR=0.58, 95% confidence interval [CI]: 0.39-0.88; Figure 19A). However, no difference was observed in the pairwise analysis of the smaller group of activity-discordant MZ pairs (HR = 1.00, 95% CI: 0.52-1.94; Figure 19B).

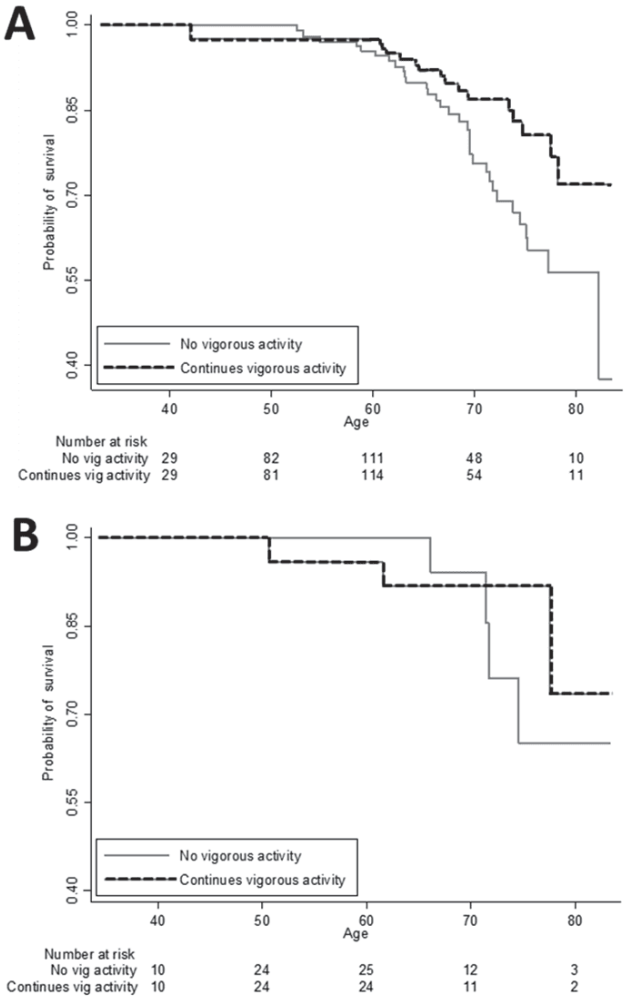


FIGURE 19 Kaplan-Meier survival curves of mortality in the human study (II). Follow-up started from the date of the 1990 questionnaire response to the end of July 2013. Groups comprised individuals with no vigorous activity (grey) vs. those with persistent vigorous activity (black dash line) at baseline (start of follow-up). (A) Survival of 134 discordant DZ twin pairs; (B) survival of 34 discordant MZ twin pairs.

6 DISCUSSION

This study investigated the role of genetic background and physical activity on skeletal muscle properties, metabolism and lifespan. The main finding was that genetic background is a stronger determinant of lifespan than physical activity level. Despite the positive effects on glucose metabolism in rodents and human twins, physical activity does not improve longevity, particularly when commenced in adulthood.

We also found that high intrinsic running capacity in rodents is associated with better fatigue tolerance and faster recovery after stimulated, maximal muscle contractions. High intrinsic aerobic capacity is also linked to higher body temperature and this trait can be maintained via voluntary running. Furthermore, we showed that high intrinsic aerobic capacity is linked to elevated SIRT3 content in skeletal muscle.

6.1 Skeletal muscle properties

At the skeletal muscle level, in the untrained state, LCRs are more sensitive to fatigue and have slower metabolic recovery compared to HCRs after maximal muscle contractions (Figure 11). A novel finding was that LCRs become more rapidly fatigued during maximal isometric muscle stimulation (Figures 11A and 12), not only during an aerobic running test, where motivation and running economy can also have a great impact on the performance. Furthermore, as we hypothesized, LCRs had poor intramuscular pH homeostasis compared to HCRs (Figure 11B).

It is likely that the slower PCr recovery of LCRs is partly due to lower mitochondrial content of the muscle compared to HCRs (Kivelä et al. 2010). Diminished oxidative capacity of the mitochondria may also play a role in the present findings; it has been shown that, despite similar mitochondrial density, LCRs have reduced mitochondrial respiratory capacity in skeletal muscle compared to HCRs (Walsh et al. 2006). The larger contractile-mediated decrease in pH in the triceps surae of LCRs was likely related to the larger glycolytic ca-

pacity in fast-twitch muscle fibers (type 2b) of LCRs compared to HCRs (Termin, Staron & Pette 1989; Fuchtbauer et al. 1991; Kivelä et al. 2010). Additionally, HCRs may have more efficient hydrogen ion buffering and a delayed decline in Ca^{2+} release due to higher oxidative capacity in skeletal muscle (Kivelä et al. 2010; Rivas et al. 2011), which might help resist fatigue.

Results from skeletal muscle protein analysis showed that HCRs had higher SIRT3 protein content in skeletal muscle compared to LCRs (Figure 13A). SIRT3 in general is linked to elevated metabolism, fatty acid oxidation and oxidative phosphorylation (Sack & Finkel 2012; Hirschey et al. 2010), which previously have been established in HCRs (Gavini et al. 2014; Novak et al. 2010; Overmyer et al. 2015; Kivelä et al. 2010).

These findings from skeletal muscle properties in the untrained state are associated with reduced running capacity along with previously found lower mitochondrial content, increased body mass and higher complex disease risk of LCRs (Kivelä et al. 2010). The higher SIRT3 level of HCRs in turn may promote increased deacetylation in mitochondria, thus, increasing the activity of enzymes of oxidative pathways. Hence, compared to LCRs, HCRs are able to more efficiently continue to produce ATP through oxidative phosphorylation, especially during exercise (Overmyer et al. 2015). As LCRs are considered as an animal model for metabolic syndrome, it is also worth noting that skeletal muscle from obese subjects or from type 2 diabetic patients' show reduced mitochondrial oxidative capacity (Mogensen et al. 2007; Kelley et al. 2002; Bakkman et al. 2010).

Aging significantly increased Hsp27, HSF1 and HSF2 levels in skeletal muscle of both HCR and LCR rats (Figure 13B-D). In normal, non-stressed cells Hsp levels are either not expressed or expressed at very low levels. Once induced, these Hsps directly modulate the execution of the apoptotic signaling pathway. Thus, Hsps have a cytoprotective function: they allow the cells to adapt to gradual changes in their environment and to survive in otherwise lethal conditions (Schmitt et al. 2007).

Aging, in general, is linked to the accumulation of oxidative stress (Wei & Lee 2002) and it also appears to attenuate the heat shock response in the myocardium of old animals (Demirel et al. 2003; Locke & Tanguay 1996). However, in skeletal muscle, old animals seem to retain their capability to accumulate Hsp protein when subjected to heat shock (Locke 2000). Both HSF1 and HSF2 are essential for protecting cells from protein-damaging stress associated with misfolded proteins and therefore aging (Ostling et al. 2007; Westerheide et al. 2009), whereas Hsp27 has been shown to increase the cellular anti-oxidant defense (Mehlen et al. 1996).

Elevated Hsp27, HSF1 and HSF2 levels reported here may indicate that muscle cells of aged HCR/LCR rats responded to aging-related accumulation of oxidative stress and attempted to compensate the stress by increasing the amount of protective molecular machinery in both of the studied rat lines (Murlasits et al. 2006). Aging also increased the level of PGC-1α and had a tendency to increase cyt c level, not showing the expected aging-related decrease

of mitochondrial function and content that has been well established previously (Figure 14) (Conley, Jubrias & Esselman 2000; Huang & Hood 2009; Johnson, Robinson & Nair 2013). According to our present results, it seems that the mitochondrial number and/or capacity in the gastrocnemius muscle were not reduced at the time point (21 months) chosen in our study. It is possible, that we would have seen the aging effect in mitochondrial markers if we had chosen a later time point.

Unfortunately the maximal isometric muscle stimulation measurements could not be repeated after one-year of the voluntary running intervention, and hence we lack the information whether voluntary running can retrieve the fast fatigability and slow recovery of LCRs. Nevertheless, one-year of voluntary running had no significant effect on the studied protein levels. It has been established in previous studies, that endurance training increases PGC-1α and cyt c levels, as well as mitochondrial content and respiratory capacity in skeletal muscle (Holloszy & Coyle 1984). Also, SIRT3 has been reported to be upregulated by exercise and chronic muscle contractions (Gurd et al. 2012, Palacios et al. 2009). It seems that in the gastrocnemius muscle, there was no stimulus to increase the markers of oxidative capacity to a significant extent, at least not at the time point chosen in our study. Moreover, PGC-1α is known to respond to exercise acutely (Baar et al. 2002). It appears in our study that the running distance decreased gradually over time (Figure 17C) concomitantly with the stimulus for adaptation.

6.2 Thermogenesis

In the untrained state, HCRs had on average 1.3°C higher body temperature compared to LCRs (mean HCR 38.0 vs LCR 36.7) ($p < 0.001$, Figure 15A). Since a normal body temperature range in rats is 35.9-37.5°C (Animal care and use committee, John Hopkins University, Baltimore, Maryland, USA), HCRs seem to have elevated body temperature compared to reference values. Nonetheless, in other animal models it has been shown that rodents with inherited obesity have low body temperatures (Levin, Comai & Sullivan 1981; Trayhurn, Thurlby & James 1977). LCRs are prone to gain excess weight and develop metabolic disorders (Noland et al. 2007; Koch & Britton 2005; Kivelä et al. 2010), and here we show for the first time that, similarly to inherited obesity, LCRs have lower body temperature than HCRs.

Aging diminished the difference in the heat generation between HCR/LCR rat lines when comparing the control groups only (HCR vs. LCR) (Figure 15B). This was due to decreased body temperature of HCRs while aging did not have a marked impact on the body temperature of LCRs. For an unknown reason, HCRs seem to lose their ability to maintain high thermogenesis during aging in control conditions (e.g. no running wheel). In a previous study, the thermogenic activity in brown adipose tissue mitochondria was greatly re-

duced in old rats, even though the UCP content in the brown adipose tissue mitochondria was similar as in young rats (Yamashita et al. 1994). In our study, we were not able to measure the thermogenic activity of brown adipose tissue or muscle tissue, which might have explained the elevated heat generation in HCRs compared to LCRs at a younger age.

As shown here and in a previous study, HCRs are spontaneously more active than LCRs (Figure 17B) and spontaneous activity also contributes to body temperature level, proposing it to be a potential cause for the higher thermogenesis of HCRs (Novak et al. 2010). However, when body temperature was measured, the total activity of the rats was very similar during both placebo and glucose tolerance test showing no effect of treatment on activity level. Nevertheless, there was a clear difference in the heat generation between the two protocols and a significant treatment effect on body temperature (Figure 16C-D, Table 2). These findings suggest that heat generation is not solely explained by spontaneous activity level.

One-year of voluntary running increased the baseline body temperature in both rat lines (Figure 15B). Further analyses showed that running had a marked impact on the body temperature of HCRs, which is in-line with their running performance compared to LCRs (Table 2). Voluntary running aids older HCRs to maintain thermogenesis at similar levels as in untrained, younger HCRs. Elevated body temperature itself may partly cause the heightened oxidative metabolism in HCRs, as enzyme-catalyzed reactions are enhanced in higher temperatures (Landsberg et al. 2009).

Our data suggests that aging does not necessarily decrease the levels of thermogenesis-related proteins, whereas heat generation at the whole-body level may be reduced. Since HCRs have more OXPHOS proteins and higher PGC-1α and UCP2 levels in skeletal muscle (Figure 14), it could be speculated that higher proton leak may also be one reason for HCRs higher thermogenesis. Previous findings have shown that female HCR have higher muscle heat dissipation during activity, explaining their low economy of activity and higher total energy expenditure compared to LCRs (Gavini et al. 2014). However, in this study critical factor was activity related thermogenesis, whereas no significant contribution of resting metabolic rate was found when body size and composition were considered (Gavini et al. 2014). Nonetheless, in our study it seems that the spontaneous activity level is not solely determining the level of thermogenesis (Figure 16). Since it is known that HCRs have higher mitochondrial content and function in skeletal muscle (Kivelä et al. 2010; Rivas et al. 2011; Walsh et al. 2006), it may be speculated that the higher thermogenesis of HCRs is an adaptation to maintain the large mitochondria reservoir viable also during resting state. On the other hand, it has been proposed that low body temperature is also one reason for the onset of obesity in humans (Landsberg et al. 2009) even though recent studies have not found a difference in the core temperatures of lean and obese subjects (Heikens et al. 2011; Hoffmann et al. 2012). Nevertheless, adult humans have substantial depots of metabolically active brown adipose tissue, suggesting that thermogenesis may play a key role in the mainte-

nance of a leaner and more metabolically healthy phenotype (Cypess et al. 2009; Virtanen et al. 2009).

However, it is worth noting that lifespan is generally negatively correlated with body temperature (Rikke & Johnson 2004). Some life-extending manipulations in rodents, such as caloric restriction, can decrease body temperature by 1-5 °C (Rikke & Johnson 2004). Furthermore, a modest prolonged reduction of core body temperature (0.3-0.5°C) increased median life expectancy in mice 12-20 % even without caloric restriction (Conti et al. 2006). Nonetheless, in the HCR/LCR animal model, HCRs have higher body temperature associated with longer lifespan than LCRs (Koch, Britton & Wisloff 2012).

The free radical hypothesis of aging nowadays includes two divergent ideas about how differences in energy metabolism might be associated with longevity. The 'rate of living-free-radical theory' suggests that higher rates of metabolism (i.e. higher free radical production) should be negatively linked to lifespan (Sohal, Mockett & Orr 2002), whereas the 'uncoupling to survive' hypothesis suggests that the correlation should be positive (Brand 2000). The latter hypothesis is based on the fact that mitochondria are incompletely coupled, dissipating part of the energy as heat (Figure 3). In a previous study, it was estimated that mitochondrial proton cycling causes up to 20-25% of basal metabolic rate in rats (Rolfe et al. 1999). It was suggested that the function of the energy-dissipating proton leak is not primarily to increase thermogenesis, but to decrease the production of ROS. Indeed, proton leak is proposed to be a key factor in aiding to decrease oxidative damage to DNA and in slowing ageing (Brand 2000, Speakman et al. 2004). It seems that HCRs are good candidates supporting the 'uncoupling to survive' hypothesis since their higher OXPHOS, PGC-1α and UCP2 levels are combined with higher thermogenesis and longer lifespan than LCRs.

Previous studies have also shown that core body temperature declines with age both in rodents and in humans (Waalen & Buxbaum 2011; Roth et al. 2002; Sanchez-Alavez, Alboni & Conti 2011), which has raised speculation of possible anti-ageing effects of low body temperature. In our studies, HCRs in the control group had lower body temperature at the 21-month time point than HCR-Rs, and this was accompanied with longer lifespan (Figures 15B, 18). According to our results it can be speculated that voluntary running may interfere with a natural aging-related reduction in body temperature of HCRs, possibly contributing to a decrease in lifespan. However, it remains controversial whether high body temperature and high rate of metabolism are beneficial to health and longevity, and more studies are needed to investigate the role of metabolic rate on aging and longevity.

6.3 Lifespan

In agreement with previous rodent studies, exercise initiated in adulthood did not reduce mortality or increase lifespan (Edington, Cosmas & McCafferty 1972; Goodrick et al. 1983) (Figures 18 and 19B). In the present study, voluntary running actually decreased lifespan by approximately 5 months in both of the studied rat lines (Figure 18). However, our results support the notion that inherited aerobic capacity is a strong determinant of longevity (Koch et al. 2011; Kujala et al. 2003).

As noted in HCR/LCR rats, the persistent discordance in participation in vigorous physical activity was significantly more common in DZ twin pairs (sharing 50% of their DNA sequence on average) than in MZ pairs (identical DNA sequence), stating that genes have an influence on the persistent voluntary participation in vigorous leisure-time physical activity. Our results indicate that genetic predisposition plays a significant role in exercise participation, which is consistent with previous data in rodents (Koch, Britton & Wisloff 2012; Kelly et al. 2010; Novak et al. 2010) and humans (Stubbe et al. 2006; de Geus et al. 2014). These results are also consistent with previous suggestion that genetic pleiotropy may partly explain the associations observed between high physical activity and mortality (Kujala et al. 2003).

High long-term participation in vigorous leisure-time physical activity did predict significantly reduced mortality in DZ twins. This finding is in agreement with epidemiological data, but causal relation between high physical activity and reduced mortality remains speculative as genetic pleiotropy may explain this association. Our findings are also consistent with previous studies that show, despite positive effects on glucose metabolism (HOMA-IR) in rodents and human twins (Rottensteiner et al. 2015; Changsun et al. 2013), that physical activity does not improve longevity in twins or rodents, particularly when commenced in maturity (Goodrick et al. 1983; Kujala, Kaprio & Koskenvuo 2002; Edington, Cosmas & McCafferty 1972).

Interestingly, in HCR/LCR rats, high endurance capacity in treadmill running test in older age (21 months) was not beneficial for longevity in either of the rat lines (Table 3). Rats in the running groups had significantly longer maximal running distances compared to the corresponding control groups, yet their lifespan was decreased (Figure 18). In contrast, during standard conditions (e.g. no voluntary running), previous findings from HCR/LCR rats show that intrinsic aerobic capacity measured as maximal oxygen consumption (VO_{2max}) assessed longitudinally at 15, 20, 25, and 34 months of age predicted lifespan both between LCR and HCR lines but also within each line (Koch et al. 2011).

Unfortunately we were not able to report the specific cause of death in our study, and this limitation impedes the speculation of the possible causes for the decreased lifespan in the runner groups. Previous study on the divide in median lifespan between the LCR and HCR rats showed no difference in incidence or severity for age-related lesions, suggesting no overt disease conditions occur-

rence in either rat line (Koch et al. 2011). From our study, deaths caused by development of spontaneous tumors with aging did not explain the group differences in lifespan (21–45% of deaths within groups).

To conclude, long-term participation in vigorous leisure-time physical activity did not predict reduced mortality in rats differing from their intrinsic aerobic capacity or in pairwise analysis among MZ twin pairs. This may have been due to the sequence-level genetic similarity, since it was difficult to find pairs who were discordant for physical activity. This finding is consistent with the lack of evidence from randomized controlled trials that high physical activity would reduce mortality in baseline healthy individuals. This result is consistent with the hypothesis that genetic pleiotropy may explain at least some of the association between high physical activity and reduced mortality. Further study, in both animals and humans, is required to determine whether longevity can be increased by exercise-enhanced aerobic capacity.

6.4 Future directions

Based on the findings of our studies I-IV, high intrinsic aerobic capacity is associated with slow fatigability and high SIRT3 protein level in skeletal muscle. At the whole-body level, high aerobic capacity is linked to high spontaneous activity and high body temperature. These traits seem to help HCRs to stay lean and healthy, thus, avoiding excess weight gain and metabolic disorders that are typical in LCRs.

The results from study I showed that low aerobic capacity was associated with fast fatigability during maximal isometric muscle stimulation. It will be of interest for future studies to investigate whether exercise training can retrieve the fast fatigability and slow recovery after maximal muscle stimulation of the LCR. Also, combining aging with physical activity would give valuable information about how much aging-related skeletal muscle function decline can be compensated via exercise in individuals with different genetic backgrounds.

At the whole-body level, for the first time our study showed that in the untrained state that HCRs have approximately 1.3°C higher body temperature compared to LCRs. This may be linked to higher proton leak, decreased oxidative damage and in slower ageing of HCRs. According to our results it can be speculated that voluntary running may interfere with the natural aging-related reduction in body temperature of HCRs, causing a decrease in lifespan. However, it is still controversial whether high body temperature and high rate of metabolism are beneficial to health and longevity. More studies investigating the effects of thermogenesis and metabolic rate on health parameters combined with longevity data need to be conducted to obtain valid data about possible associations between these parameters. Nevertheless, as more evidence of obesity being associated with low body temperature in rodents emerges, it would

be worth studying the potential role of intrinsically low body temperature at the onset of obesity in humans.

As noted both in previous studies and our present study, HCRs are spontaneously more active compared to LCRs. Our twin study also confirmed earlier assumptions that genes have an influence on the participation in vigorous leisure-time physical activity in humans. However, based on our findings and previous literature, exercise initiated in adulthood did not reduce mortality or increase lifespan in rodents or identical twins. In the future, to obtain more knowledge about how genetics and physical activity affect lifespan, large scale, randomized controlled intervention trials should be carried out. Combining data from several twins cohorts with both physical activity questionnaires and objective measurements (e.g. accelerometers, heart-rate measurements, VO_{2max}) starting from childhood would provide more specific answers on how intrinsic and adaptive endurance capacity affect lifespan.

Also, more studies are needed to determine whether physical activity affects lifespan differently when started early in life compared to starting later in adult life. As our study provided the first evidence of a possible link between elevated SIRT3 protein levels and longevity, it would be of common interest to investigate whether high SIRT3 expression is also linked to long lifespan in humans.

In the future, sequencing whole-genomes of large cohorts together with physical activity and longevity data enables studying which particular genes/gene sets are responsible for high endurance capacity, high physical activity level and longevity and to what extent these genes are interconnected.

7 MAIN FINDINGS AND CONCLUSIONS

The main results and conclusions of this thesis are summarized as follows:

1. In the untrained state, low intrinsic aerobic capacity was associated with fast muscular fatigue and slow metabolic recovery after maximal muscle contractions. It seems that the lower content and/or reduced respiratory capacity of mitochondria together with a larger relative amount of type 2b glycolytic muscle fibers contribute to these undesirable characteristics of skeletal muscle of LCRs. At the skeletal muscle protein level, this study showed a direct link between elevated SIRT3 protein levels and high intrinsic aerobic capacity, which may be associated with an extended lifespan in mammals. At the whole-body level, LCRs had lower body temperature compared to HCRs, similarly as in animal models of inherited obesity. The lower body temperature of LCRs may play a role in the onset of gaining extra weight and, thus, developing metabolic disorders.

2. Aging significantly increased Hsp27, HSF1 and HSF2 levels in skeletal muscle in both HCR and LCR rats. Elevated heat-shock protein levels may indicate that muscle cells of aged animals responded to aging-related accumulation of oxidative stress and attempted to compensate the stress by increasing the amount of protective molecular machinery. Interestingly, aging diminished the difference in body temperature between HCR/LCR rats. This was due to decreased body temperature of HCRs; for an unknown reason, HCRs seem to lose their ability to maintain high thermogenesis with aging in control conditions.

3. One-year voluntary running also aided HCRs to maintain thermogenesis to similar levels as in untrained, younger HCRs. This result suggests that voluntary running is crucial in maintaining the high levels of thermogenesis that, in turn, may play a key role in the maintenance of a leaner and more metabolically healthy phenotype in HCRs.

4. Vigorous physical activity initiated in adulthood did not reduce mortality or increase lifespan, neither in rodents nor in human MZ twins; however, intrinsic aerobic capacity was a strong determinant of longevity. High long-term participation in vigorous leisure-time physical activity did predict significantly reduced mortality in DZ twins; however, there was no difference in the lifespan of MZ twins. We also confirmed earlier findings, that HCRs were more physically active both in control and voluntary running groups when compared to corresponding LCR groups. Also, the persistent discordances in participation of vigorous physical activity were significantly more common in DZ twin pairs than in MZ pairs, stating that genes have an influence on persistent voluntary participation in vigorous leisure-time physical activity. Our results indicated that genetic predisposition plays a significant role in exercise participation, and genetic pleiotropy may partly explain the associations observed between high physical activity and mortality.

YHTEENVETO (FINNISH SUMMARY)

Fyysinen aktiivisuus ja aerobinen suorituskyky vaikuttavat merkittävästi yksilön terveyteen ja elinikään. Nykyinen ympäristö kuitenkin suosii inaktiivisia elämätapoja, ja sen seurauksena liikalihavuus ja siihen liittyvät sairaudet, kuten metabolinen oireyhtymä ja tyypin 2 diabetes, yleistyvät maailmanlaajuisesti kiihtyvää vauhtia. Ylipainon liitännäissairaudet ovat suuri terveydellinen ja taloudellinen uhka, ja lisäksi nämä sairaudet vaikuttavat jo nykyiseen elinajanodotteeseen. Kuitenkin osa ihmisistä kykenee välttämään ylipainon kehittymisen vaikka elinolot sitä suosisivat. Tiedemaailmassa onkin herännyt kiinnostus selvittää perimän ja fyysisen aktiivisuuden vaikutuksia elinikään.

Aiemmissa tutkimuksissa on havaittu vahva yhteys yksilön aerobisen suorituskyvyn ja eliniän välillä. Aerobista suorituskykyä pystytään mittaamaan laboratoriossa maksimaalisen hapenottokyvyn avulla. Maksimaalinen hapenottokyky mittaa hengitys- ja verenkiertoelimistön kykyä kuljettaa happea ja lihasten kykyä käyttää sitä energiantuotantoon maksimaalisessa rasituksessa. Koe-eläimillä on havaittu, että perinnöllisesti korkea aerobinen suorituskyky on yhteydessä terveyteen ja pitkään elinikään jopa ilman varsinaista fyysistä harjoittelua. Sitä vastoin perinnöllisesti matala aerobinen suorituskyky on yhteydessä ylipainoon, suurentuneeseen metabolisten ja sydän- ja verisuonitautien riskiin sekä lyhentyneeseen elinikään.

Fyysinen harjoittelu nostaa yksilön aerobista suorituskykyä - tämän vuoksi onkin tärkeää selvittää fyysisen harjoittelun vaikutusta elinikään. Aiemmissa tutkimuksissa on havaittu, että fyysisesti aktiiviset ihmiset ovat terveempiä ja elävät pidempään verrattuna vähemmän liikkuviin ihmisiin. Fyysisen aktiivisuuden määrä ja kiinnostus liikkua vapaa-ajalla kuitenkin vaihtelevat yksilöiden välillä suuresti. Viimeaikaiset tutkimukset ovatkin antaneet viitteitä siitä, että geeniperimällä on merkittävä vaikutus yksilön fyysisen aktiivisuuden määrään. Koe-eläimillä on jo pystytty osoittamaan, että geeniperimällä ja fyysisellä aktiivisuudella on selvä yhteys. Koska liikuntaa suositellaan kaikille riippumatta geeniperimästä, on tärkeää tutkia fyysisen aktiivisuuden vaikutuksia luustolihaksen ominaisuuksiin, metaboliaan ja elinikään yksilöillä, jotka eroavat toisistaan geneettiseltä taustaltaan.

Koe-eläimillä on mahdollista tutkia ilman harjoittelua hankittua, synnynnäistä aerobista suorituskykyä. Tähän tarkoitukseen on kehitetty kaksi eri linjaa, jotka eroavat perinnölliseltä kestävyysjuoksukyvyltään: HCR (high-capacity runner) ja LCR (low-capacity runner). HCR-rotilla on korkea aerobinen suorituskyky, ne ovat fyysisesti aktiivisia, terveitä ja elävät pidempään verrattuna LCR-rottiin. LCR-rotat lihovat helposti ja niillä on suurentunut riski sairastua metabolisiin sairauksiin. Ihmisillä geeniperimän ja liikunnan vaikutusta pystytään tutkimaan kaksosasetelmalla. Tutkimuksissa käytetään sekä epäidenttisiä että identtisiä kaksospareja.

Tämän väitöskirjatyön tarkoituksena oli tutkia geneettisen taustan, ikääntymisen ja fyysisen aktiivisuuden vaikutuksia luustolihaksen ominaisuuksiin, koko kehon metaboliaan ja elinikään. Väitöskirjatyö koostui kolmesta eri osa-

tutkimuksesta: (1) HCR/LCR rottien poikittaisesta juoksuinterventiokokeesta, (2) HCR/LCR -rottien pitkittäisestä juoksuinterventiokokeesta (3) pitkittäisestä seurantatutkimuksesta kaksosilla. HCR/LCR -rottien poikittaisessa tutkimuksessa kerättiin tietoa perinnöllisen aerobisen suorituskyvyn vaikutuksesta luustolihaksen ominaisuuksiin ilman liikunnan vaikutuksia. Tämän jälkeen rotat jaettiin kontrolli (tavallinen rottahäkki) ja juoksijaryhmiin (häkeissä juoksupyörä vapaaehtoista juoksuharjoittelua varten). Seurantanäytteet kerättiin vuosi juoksuintervention aloittamisen jälkeen. Pitkittäisessä HCR/LCR -rottien juoksuinterventiokokeessa tutkittiin juoksemisen ja fyysisen aktiivisuuden vaikutusta elinikään. Kaksosilla tehdyssä seurantatutkimuksessa tutkittiin samaa sukupuolta olevia epäidenttisiä ja identtisiä kaksospareja, jotka erosivat toisistaan liikuntatottumuksiltaan. Tutkimuksessa selvitettiin fyysisen aktiivisuuden ja eliniän yhteyttä keräämällä tulokset kaksosten eliniästä 23 vuoden ajalta.

Tutkimuksissa havaittiin, että rotilla perinnöllisesti matala aerobinen suoristuskyky on yhteydessä luustolihaksen nopeaan väsymiseen ja hitaaseen palautumiseen maksimaalisten lihassupistusten jälkeen. Luustolihastasolla havaittiin myös suora yhteys kohonneen SIRT3-proteiinitason ja korkean aerobisen suorituskyvyn välillä, mikä voi puolestaan olla yhteydessä pitkään elinikään. Koko kehon tasolla alhainen aerobinen suorituskyky oli yhteydessä matalaan kehonlämpöön. Sama ilmiö on aiemmin havaittu perinnöllisesti liikalihavilta eläimiltä. Matala kehonlämpö voi osaltaan vaikuttaa LCR:lle tyypillisen ylimääräisen painonnousun toimien näin yhtenä tekijänä ylipainon laukaisussa ja metabolisten sairauksien kehittymisessä.

Aikuisiässä aloitettu fyysinen aktiivisuus ei vähentänyt kuolleisuutta tai lisännyt elinikää rotilla tai identtisillä kaksosilla. Kuten aiemmissa tutkimuksissa oli havaittu, yksilön aerobinen suorituskyky kuitenkin ennusti elinikää hyvin. Korkea fyysinen aktiivisuus oli yhteydessä pitkään elinikään, kun tarkasteltiin geeniperimältään eroavia, epäidenttisiä kaksospareja. Tarkasteltaessa identtisiä kaksosia, joilla geeniperimä on sama, tätä eroa ei nähty. Tuloksemme vahvistivat aiemman havainnon, että HCR-rotat ovat selvästi LCR-rottia aktiivisempia liikkumaan häkissä niin spontaanisti kuin juoksupyörässäkin. Vastaava ilmiö havaittiin kaksosilla; liikuntatottumuksilta eroaminen oli huomattavasti yleisempää epäidenttisillä kaksosilla verrattaessa identtisiin kaksosiin.

Tämä väitöskirja osoittaa, että perinnöllisesti korkea aerobinen suorituskyky on yhteydessä luustolihaksen hyvään väsymisensietokykyyn, nopeaan palautumiseen sekä korkeaan ikääntymiseen liittyvän SIRT3-proteiinin tasoon luustolihaksessa. Sen sijaan perinnöllisesti matala aerobinen suorituskyky on yhteydessä matalaan kehonlämpöön, joka voi osaltaan johtaa ylipainon ja sen liitännäissairauksien kehittymiseen. Tutkimuksemme koe-eläin mallilla ja kaksosilla antaa viitteitä siitä, että geeniperimällä on selvä vaikutus liikuntatottumuksiin. Tulostemme perusteella geneettinen vaihtelu voi osaltaan selittää aiemmissa tutkimuksissa havaittua yhteyttä liikunnan ja pitkän eliniän välillä.

REFERENCES

Alexander, M., Petri, H., Ding, Y., Wandel, C., Khwaja, O. & Foskett, N. 2015. Morbidity and medication in a large population of individuals with Down syndrome compared to the general population. Developmental Medicine and Child Neurology 58(3), 246-254.

Allen, D. G., Westerblad, H., Lee, J. A. & Lannergren, J. 1992. Role of excitation-contraction coupling in muscle fatigue. Sports Medicine 13 (2), 116-126.

Andersen, L. B., Schnohr, P., Scroll, M. & Hein, H. O. 2002. Mortality associated with physical activity in leisure time, at work, in sports and cycling to work. Ugeskrift for Laeger 164 (11), 1501-1506.

Anderson, R. & Prolla, T. 2009. PGC-1alpha in aging and anti-aging interventions. Biochimica et Biophysica Acta 1790 (10), 1059-1066.

Åstrand, I. 1960. Aerobic capacity in men and women with specific reference to age. Acta Physiologica Scandinavica Supplement 49, 1-92.

Baar, K., Wende, A. R., Jones, T. E., Marison, M., Nolte, L. A., Chen, M., Kelly, D. P. & Holloszy, J. O. 2002. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB journal 16 (14), 1879-1886.

Baggish, A. L. & Wood, M. J. 2011. Athlete's heart and cardiovascular care of the athlete: scientific and clinical update. Circulation 123 (23), 2723-2735.

Bakkman, L., Fernstrom, M., Loogna, P., Rooyackers, O., Brandt, L. & Lagerros, Y. T. 2010. Reduced respiratory capacity in muscle mitochondria of obese subjects. Obesity Facts 3 (6), 371-375.

Bassett, D. R.,Jr & Howley, E. T. 2000. Limiting factors for maximum oxygen uptake and determinants of endurance performance. Medicine and Science in Sports and Exercise 32 (1), 70-84.

Benedict, G. & Sherman, H. C. 1937. Basal metabolism of rats in relation to old age and exercise during old age. Journal of Nutrition 14, 179-198.

Bishop, N. A. & Guarente, L. 2007. Genetic links between diet and lifespan: shared mechanisms from yeast to humans. Nature Reviews, Genetics 8 (11), 835-844.

Blair, S. N., Cheng, Y. & Holder, J. S. 2001. Is physical activity or physical fitness more important in defining health benefits? Medicine and Science in Sports and Exercise 33 (6 Suppl), S379-99; discussion S419-20.

Blomstrand, E., Radegran, G. & Saltin, B. 1997. Maximum rate of oxygen uptake by human skeletal muscle in relation to maximal activities of enzymes in the Krebs cycle. The Journal of Physiology 501(Pt 2), 455-460.

Bogdanis, G. C. 2012. Effects of physical activity and inactivity on muscle fatigue. Frontiers in Physiology 18 (3), 142.

Boomsma, D., Busjahn, A. & Peltonen, L. 2002. Classical twin studies and beyond. Nature Reviews. Genetics 3 (11), 872-882.

Bouchard, C., Dionne, F. T., Simoneau, J. A. & Boulay, M. R. 1992. Genetics of aerobic and anaerobic performances. Exercise and Sport Sciences Reviews 20, 27-58.

Bouchard, C., Rankinen, T., Chagnon, Y. C., Rice, T., Perusse, L., Gagnon, J., Borecki, I., An, P., Leon, A. S., Skinner, J. S., Wilmore, J. H., Province, M. & Rao, D. C. 2000. Genomic scan for maximal oxygen uptake and its response to training in the HERITAGE Family Study. Journal of Applied Physiology 88 (2), 551-559.

Brand, M. D. 2000. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Experimental Gerontology 35 (6-7), 811-820.

Bray, M. S. 2000. Genomics, genes, and environmental interaction: the role of exercise. Journal of Applied Physiology 88 (2), 788-792.

Burgomaster, K. A., Cermak, N. M., Phillips, S. M., Benton, C. R., Bonen, A. & Gibala, M. J. 2007. Divergent response of metabolite transport proteins in human skeletal muscle after sprint interval training and detraining. American Journal of Physiology 292 (5), R1970-1976.

Burke, R. E., Levine, D. N., Tsairis, P. & Zajac, F. E.,3rd 1973. Physiological types and histochemical profiles in motor units of the cat gastrocnemius. The Journal of Physiology 234 (3), 723-748.

Buskirk, E. R. & Hodgson, J. L. 1987. Age and aerobic power: the rate of change in men and women. Federation Proceedings 46 (5), 1824-1829.

Cacho, J., Sevillano, J., de Castro, J., Herrera, E. & Ramos, M. P. 2008. Validation of simple indexes to assess insulin sensitivity during pregnancy in Wistar and Sprague-Dawley rats. American Journal of Physiology 295 (5), E1269-1276.

Cannon, B. & Nedergaard, J. 2004. Brown adipose tissue: function and physiological significance. Physiological Reviews 84 (1), 277-359.

Capelli, C., Antonutto, G., Kenfack, M. A., Cautero, M., Lador, F., Moia, C., Tam, E. & Ferretti, G. 2006. Factors determining the time course of VO2(max) decay during bedrest: implications for VO2(max) limitation. European Journal of Applied Physiology 98 (2), 152-160.

Carlborg, O., Jacobsson, L., Ahgren, P., Siegel, P. & Andersson, L. 2006. Epistasis and the release of genetic variation during long-term selection. Nature Genetics 38 (4), 418-420.

Carroll, T. J., Riek, S. & Carson, R. G. 2002. The sites of neural adaptation induced by resistance training in humans. The Journal of Physiology 544 (Pt 2), 641-652.

Changsun, K., Jinhwan, Y., Dongho, P., Jiyeon, K., Hyojin, K. & Sangki, L. 2013. The effects of aerobic treadmill exercise training on insulin resistance and bone metabolic turnover in diabetes mellitus rats. Journal of Exercise Nutrition Biochemistry 17 (3), 61-69.

Choi, C. S., Fillmore, J. J., Kim, J. K., Liu, Z. X., Kim, S., Collier, E. F., Kulkarni, A., Distefano, A., Hwang, Y. J., Kahn, M., Chen, Y., Yu, C., Moore, I. K., Reznick, R. M., Higashimori, T. & Shulman, G. I. 2007. Overexpression of uncoupling protein 3 in skeletal muscle protects against fat-induced insulin resistance. The Journal of Clinical Investigation 117 (7), 1995-2003.

Conley, K. E., Jubrias, S. A. & Esselman, P. C. 2000. Oxidative capacity and ageing in human muscle. The Journal of Physiology 526 (Pt 1), 203-210.

Conti, B., Sanchez-Alavez, M., Winsky-Sommerer, R., Morale, M. C., Lucero, J., Brownell, S., Fabre, V., Huitron-Resendiz, S., Henriksen, S., Zorrilla, E. P., de Lecea, L. & Bartfai, T. 2006. Transgenic mice with a reduced core body temperature have an increased life span. Science 314 (5800), 825-828.

Corder, E. H., Lannfelt, L., Viitanen, M., Corder, L. S., Manton, K. G., Winblad, B. & Basun, H. 1996. Apolipoprotein E genotype determines survival in the oldest old (85 years or older) who have good cognition. Archives of Neurology 53 (5), 418-422.

Costford, S. R., Chaudhry, S. N., Crawford, S. A., Salkhordeh, M. & Harper, M. E. 2008. Long-term high-fat feeding induces greater fat storage in mice lacking UCP3. American Journal of Physiology 295 (5), E1018-1024.

Cureton, K., Bishop, P., Hutchinson, P., Newland, H., Vickery, S. & Zwiren, L. 1986. Sex difference in maximal oxygen uptake. Effect of equating haemoglobin concentration. European Journal of Applied Physiology and Occupational Physiology 54 (6), 656-660.

Cypess, A. M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A. B., Kuo, F. C., Palmer, E. L., Tseng, Y. H., Doria, A., Kolodny, G. M. & Kahn, C. R. 2009. Identification and importance of brown adipose tissue in adult humans. The New England Journal of Medicine 360 (15), 1509-1517.

De Benedictis, G., Rose, G., Carrieri, G., De Luca, M., Falcone, E., Passarino, G., Bonafe, M., Monti, D., Baggio, G., Bertolini, S., Mari, D., Mattace, R. & Franceschi, C. 1999. Mitochondrial DNA inherited variants are associated with successful aging and longevity in humans. FASEB journal 13 (12), 1532-1536.

de Geus, E. J., Bartels, M., Kaprio, J., Lightfoot, J. T. & Thomis, M. 2014. Genetics of regular exercise and sedentary behaviors. Twin Research and Human Genetics 17 (4), 262-271.

Demirel, H. A., Hamilton, K. L., Shanely, R. A., Tumer, N., Koroly, M. J. & Powers, S. K. 2003. Age and attenuation of exercise-induced myocardial HSP72 accumulation. American Journal of Physiology 285 (4), H1609-1615.

Demontis, F., Piccirillo, R., Goldberg, A. L. & Perrimon, N. 2013. The influence of skeletal muscle on systemic aging and lifespan. Aging cell 12 (6), 943-949.

Drinkwater, B. L. 1984. Women and exercise: physiological aspects. Exercise and Sport Sciences Reviews 12, 21-51.

Edington, D. W., Cosmas, A. C. & McCafferty, W. B. 1972. Exercise and longevity: Evidence for a threshold age. Journal of Gerontology 27, 341.

Edstrom, L. & Kugelberg, E. 1968. Histochemical composition, distribution of fibres and fatiguability of single motor units. Anterior tibial muscle of the rat. Journal of Neurology, Neurosurgery, and Psychiatry 31 (5), 424-433.

Engstrom, L. M. & Fischbein, S. 1977. Physical capacity in twins. Acta Geneticae Medicae et Gemellologiae 26 (2), 159-165.

Fabris, N. 1991. Neuroendocrine-immune interactions: a theoretical approach to aging. Archives of Gerontology and Geriatrics 12 (2-3), 219-230.

Fridovich, I. 1997. Superoxide anion radical (O2-.), superoxide dismutases, and related matters. The Journal of Biological Chemistry 272 (30), 18515-18517.

Frisancho, A. R., Frisancho, H. G., Milotich, M., Brutsaert, T., Albalak, R., Spielvogel, H., Villena, M., Vargas, E. & Soria, R. 1995. Developmental, genetic, and environmental components of aerobic capacity at high altitude. American Journal of Physical Anthropology 96 (4), 431-442.

Fuchi, T., Iwaoka, K., Higuchi, M. & Kobayashi, S. 1989. Cardiovascular changes associated with decreased aerobic capacity and aging in long-distance runners. European Journal of Applied Physiology and Occupational Physiology 58 (8), 884-889.

Fuchtbauer, E. M., Rowlerson, A. M., Gotz, K., Friedrich, G., Mabuchi, K., Gergely, J. & Jockusch, H. 1991. Direct correlation of parvalbumin levels with myosin isoforms and succinate dehydrogenase activity on frozen sections of rodent muscle. The Journal of Histochemistry and Cytochemistry 39 (3), 355-361.

Garcia-Valles, R., Gomez-Cabrera, M. C., Rodriguez-Manas, L., Garcia-Garcia, F. J., Diaz, A., Noguera, I., Olaso-Gonzalez, G. & Vina, J. 2013. Life-long spontaneous exercise does not prolong lifespan but improves health span in mice. Longevity & Healthspan 2 (1), 14-2395-2-14.

Gavini, C. K., Mukherjee, S., Shukla, C., Britton, S. L., Koch, L. G., Shi, H. & Novak, C. M. 2014. Leanness and heightened nonresting energy expenditure: role of skeletal muscle activity thermogenesis. American Journal of Physiology 306 (6), E635-647.

Geiser, F. 1988. Reduction of metabolism during hibernation and daily torpor in mammals and birds: temperature effect or physiological inhibition? Journal of Comparative Physiology 158 (1), 25-37.

George, K. P., Wolfe, L. A., Burggraf, G. W. & Norman, R. 1995. Electrocardiographic and echocardiographic characteristics of female athletes. Medicine and Science in Sports and Exercise 27 (10), 1362-1370.

Giannesini, B., Izquierdo, M., Le Fur, Y., Cozzone, P. J. & Bendahan, D. 2001. In vivo reduction in ATP cost of contraction is not related to fatigue level in stimulated rat gastrocnemius muscle. The Journal of Physiology 536 (Pt 3), 905-915.

Giannesini, B., Izquierdo, M., Le Fur, Y., Cozzone, P. J., Fingerle, J., Himber, J., Kunnecke, B., Von Kienlin, M. & Bendahan, D. 2005. New experimental setup for studying strictly noninvasively skeletal muscle function in rat using 1H-magnetic resonance (MR) imaging and 31P-MR spectroscopy. Magnetic Resonance in Medicine 54 (5), 1058-1064.

Gonzalez, N. C., Howlett, R. A., Henderson, K. K., Koch, L. G., Britton, S. L., Wagner, H. E., Favret, F. & Wagner, P. D. 2006. Systemic oxygen transport in rats artificially selected for running endurance. Respiratory Physiology & Neurobiology 151 (2-3), 141-150.

Gonzalez-Alonso, J. & Calbet, J. A. 2003. Reductions in systemic and skeletal muscle blood flow and oxygen delivery limit maximal aerobic capacity in humans. Circulation 107 (6), 824-830.

Goodrick, C. L. 1980. Effects of long-term voluntary wheel exercise on male and female Wistar rats. I. Longevity, body weight, and metabolic rate. Gerontology 26 (1), 22-33.

Goodrick, C. L., Ingram, D. K., Reynolds, M. A., Freeman, J. R. & Cider, N. L. 1983. Differential effects of intermittent feeding and voluntary exercise on body weight and lifespan in adult rats. Journal of Gerontology 38 (1), 36-45.

Gudmundsson, H., Gudbjartsson, D. F., Frigge, M., Gulcher, J. R. & Stefansson, K. 2000. Inheritance of human longevity in Iceland. European Journal of Human Genetics 8 (10), 743-749.

Gurd, B. J., Holloway, G. P., Yoshida, Y. & Bonen, A. 2012. In mammalian muscle, SIRT3 is present in mitochondria and not in the nucleus; and SIRT3 is upregulated by chronic muscle contraction in an adenosine monophosphate-activated protein kinase-independent manner. Metabolism: Clinical and Experimental 61 (5), 733-741.

Hagberg, J. M. 1987. Effect of training on the decline of VO2max with aging. Federation Proceedings 46 (5), 1830-1833.

Hamel, P., Simoneau, J. A., Lortie, G., Boulay, M. R. & Bouchard, C. 1986. Heredity and muscle adaptation to endurance training. Medicine and Science in Sports and Exercise 18 (6), 690-696.

Hawkins, S. & Wiswell, R. 2003. Rate and mechanism of maximal oxygen consumption decline with aging: implications for exercise training. Sports Medicine 33 (12), 877-888.

Hawkins, S. A., Marcell, T. J., Victoria Jaque, S. & Wiswell, R. A. 2001. A longitudinal assessment of change in VO2max and maximal heart rate in master athletes. Medicine and Science in Sports and Exercise 33 (10), 1744-1750.

Heikens, M. J., Gorbach, A. M., Eden, H. S., Savastano, D. M., Chen, K. Y., Skarulis, M. C. & Yanovski, J. A. 2011. Core body temperature in obesity. The American Journal of Clinical Nutrition 93 (5), 963-967.

Herskind, A. M., McGue, M., Holm, N. V., Sorensen, T. I., Harvald, B. & Vaupel, J. W. 1996. The heritability of human longevity: a population-based study of 2872 Danish twin pairs born 1870-1900. Human Genetics 97 (3), 319-323.

Hickson, R. C. 1981. Skeletal muscle cytochrome c and myoglobin, endurance, and frequency of training. Journal of Applied Physiology 51 (3), 746-749.

Hickson, R. C., Hagberg, J. M., Ehsani, A. A. & Holloszy, J. O. 1981. Time course of the adaptive responses of aerobic power and heart rate to training. Medicine and Science in Sports and Exercise 13 (1), 17-20.

Hirschey, M. D., Shimazu, T., Goetzman, E., Jing, E., Schwer, B., Lombard, D. B., Grueter, C. A., Harris, C., Biddinger, S., Ilkayeva, O. R., Stevens, R. D., Li, Y., Saha, A. K., Ruderman, N. B., Bain, J. R., Newgard, C. B., Farese, R. V.,Jr, Alt, F. W., Kahn, C. R. & Verdin, E. 2010. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature 464 (7285), 121-125.

Hoffmann, M. E., Rodriguez, S. M., Zeiss, D. M., Wachsberg, K. N., Kushner, R. F., Landsberg, L. & Linsenmeier, R. A. 2012. 24-H Core Temperature in Obese and Lean Men and Women. Obesity 20 (8), 1585-1590.

Holloszy, J. O. 1998. Longevity of exercising male rats: effect of an antioxidant supplemented diet. Mechanisms of Ageing and Development 100 (3), 211-219.

Holloszy, J. O. 1993. Exercise increases average longevity of female rats despite increased food intake and no growth retardation. Journal of Gerontology 48 (3), B97-100.

Holloszy, J. O. 1967. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. The Journal of Biological Chemistry 242 (9), 2278-2282.

Holloszy, J. O. & Coyle, E. F. 1984. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. Journal of Applied Physiology 56 (4), 831-838.

Holloszy, J. O., Smith, E. K., Vining, M. & Adams, S. 1985. Effect of voluntary exercise on longevity of rats. Journal of Applied Physiology 59 (3), 826-831.

Howald, H. 1976. Ultrastructure and biochemical function of skeletal muscle in twins. Annals of Human Biology 3 (5), 455-462.

Huang, J. H. & Hood, D. A. 2009. Age-associated mitochondrial dysfunction in skeletal muscle: Contributing factors and suggestions for long-term interventions. IUBMB life 61 (3), 201-214.

Jacobs, D. R.,Jr, Ainsworth, B. E., Hartman, T. J. & Leon, A. S. 1993. A simultaneous evaluation of 10 commonly used physical activity questionnaires. Medicine and Science in Sports and Exercise 25 (1), 81-91.

Jazwinski, S. M. 2000. Aging and longevity genes. Acta Biochimica Polonica 47 (2), 269-279.

Johnsen, A. B., Rolim, N. P., Stolen, T., Alves, M., Sousa, M. M., Slupphaug, G., Britton, S. L., Koch, L. G., Smith, G. L., Wisloff, U. & Hoydal, M. A. 2013. Atrial myocyte function and Ca2+ handling is associated with inborn aerobic capacity. PloS One 8 (10), e76568.

Johnson, M. L., Robinson, M. M. & Nair, K. S. 2013. Skeletal muscle aging and the mitochondrion. Trends in Endocrinology and Metabolism 24 (5), 247-256.

Jones, A. M. 1998. A five year physiological case study of an Olympic runner. British Journal of Sports Medicine 32 (1), 39-43.

Jones, A. M. & Carter, H. 2000. The effect of endurance training on parameters of aerobic fitness. Sports Medicine 29 (6), 373-386.

Kaeberlein, M., McVey, M. & Guarente, L. 1999. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes & Development 13 (19), 2570-2580.

Kaprio, J. & Koskenvuo, M. 2002. Genetic and environmental factors in complex diseases: the older Finnish Twin Cohort. Twin Research 5 (5), 358-365.

Kelley, D. E., He, J., Menshikova, E. V. & Ritov, V. B. 2002. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes 51 (10), 2944-2950.

Kelly, S. A., Nehrenberg, D. L., Peirce, J. L., Hua, K., Steffy, B. M., Wiltshire, T., Pardo-Manuel de Villena, F., Garland, T.,Jr & Pomp, D. 2010. Genetic architecture of voluntary exercise in an advanced intercross line of mice. Physiological Genomics 42 (2), 190-200.

Kemi, O. J., Haram, P. M., Loennechen, J. P., Osnes, J. B., Skomedal, T., Wisloff, U. & Ellingsen, O. 2005. Moderate vs. high exercise intensity: differential effects on aerobic fitness, cardiomyocyte contractility, and endothelial function. Cardiovascular Research 67 (1), 161-172.

Kivelä, R., Silvennoinen, M., Lehti, M., Rinnankoski-Tuikka, R., Purhonen, T., Ketola, T., Pullinen, K., Vuento, M., Mutanen, N., Sartor, M. A., Reunanen, H., Koch, L. G., Britton, S. L. & Kainulainen, H. 2010. Gene expression centroids that link with low intrinsic aerobic exercise capacity and complex disease risk. The FASEB journal 24 (11), 4565-4574.

Koch, L. G. & Britton, S. L. 2005. Divergent selection for aerobic capacity in rats as a model for complex disease. Integrative and Comparative Biology 45 (3), 405-415.

Koch, L. G. & Britton, S. L. 2001. Artificial selection for intrinsic aerobic endurance running capacity in rats. Physiological Genomics 5 (1), 45-52.

Koch, L. G., Britton, S. L. & Wisloff, U. 2012. A rat model system to study complex disease risks, fitness, aging, and longevity. Trends in Cardiovascular Medicine 22 (2), 29-34.

Koch, L. G., Kemi, O. J., Qi, N., Leng, S. X., Bijma, P., Gilligan, L. J., Wilkinson, J. E., Wisloff, H., Hoydal, M. A., Rolim, N., Abadir, P. M., Van Grevenhof, I., Smith, G. L., Burant, C. F., Ellingsen, O., Britton, S. L. & Wisloff, U. 2011. Intrinsic Aerobic Capacity Sets a Divide for Aging and Longevity. Circulation Research 28;109(10):1162-1172.

Koch, L. G., Pollott, G. E. & Britton, S. L. 2013. Selectively bred rat model system for low and high response to exercise training. Physiological Genomics 45 (14), 606-614.

Kodama, S., Saito, K., Tanaka, S., Maki, M., Yachi, Y., Asumi, M., Sugawara, A., Totsuka, K., Shimano, H., Ohashi, Y., Yamada, N. & Sone, H. 2009. Cardiorespiratory fitness as a quantitative predictor of all-cause mortality and cardiovascular events in healthy men and women: a meta-analysis. Journal of the American Medical Association 301 (19), 2024-2035.

Kruk, P. A., Rampino, N. J. & Bohr, V. A. 1995. DNA damage and repair in telomeres: relation to aging. Proceedings of the National Academy of Sciences of the United States of America 92 (1), 258-262.

Kujala UM, Kaprio J, Sarna S,Koskenvuo M. 1998. Relationship of leisure-time physical activity and mortality: The Finnish twin cohort. Journal of the American Medical Association 279 (6), 440-444.

Kujala, U. M., Kaprio, J. & Koskenvuo, M. 2002. Modifiable risk factors as predictors of all-cause mortality: the roles of genetics and childhood environment. American Journal of Epidemiology 156 (11), 985-993.

Kujala, U. M., Kaprio, J., Sarna, S. & Koskenvuo, M. 1998. Relationship of leisure-time physical activity and mortality: the Finnish twin cohort. Journal of the American Medical Assiciation 279 (6), 440-444.

Kujala, U. M., Marti, P., Kaprio, J., Hernelahti, M., Tikkanen, H. & Sarna, S. 2003. Occurrence of chronic disease in former top-level athletes. Predominance of benefits, risks or selection effects? Sports Medicine 33 (8), 553-561.

Landsberg, L. 2012. Core temperature: a forgotten variable in energy expenditure and obesity? Obesity Reviews: 13 Suppl 2, 97-104.

Landsberg, L., Young, J. B., Leonard, W. R., Linsenmeier, R. A. & Turek, F. W. 2009. Is obesity associated with lower body temperatures? Core temperature: a forgotten variable in energy balance. Metabolism: Clinical and Experimental 58 (6), 871-876.

Lee, D. C., Sui, X., Ortega, F. B., Kim, Y. S., Church, T. S., Winett, R. A., Ekelund, U., Katzmarzyk, P. T. & Blair, S. N. 2011. Comparisons of leisure-time physical activity and cardiorespiratory fitness as predictors of all-cause mortality in men and women. British Journal of Sports Medicine 45 (6), 504-510.

Legaz Arrese, A., Serrano Ostariz, E., Jcasajus Mallen, J. A. & Munguia Izquierdo, D. 2005. The changes in running performance and maximal oxygen uptake after long-term training in elite athletes. The Journal of Sports Medicine and Physical Fitness 45 (4), 435-440.

Leibovitz, B. E. & Siegel, B. V. 1980. Aspects of free radical reactions in biological systems: aging. Journal of Gerontology 35 (1), 45-56.

Levin, B. E., Comai, K. & Sullivan, A. C. 1981. Metabolic and sympatho-adrenal abnormalities in the obese Zucker rat: effect of chronic phenoxybenzamine treatment. Pharmacology, Biochemistry, and Behavior 14 (4), 517-525.

Licastro, F., Candore, G., Lio, D., Porcellini, E., Colonna-Romano, G., Franceschi, C. & Caruso, C. 2005. Innate immunity and inflammation in ageing: a key for understanding age-related diseases. Immunity & Ageing: 18 (2), 8.

Locke, M. 2000. Heat shock transcription factor activation and hsp72 accumulation in aged skeletal muscle. Cell Stress & Chaperones 5 (1), 45-51.

Locke, M. & Tanguay, R. M. 1996. Diminished heat shock response in the aged myocardium. Cell Stress & Chaperones 1 (4), 251-260.

Loe, H., Rognmo, O., Saltin, B. & Wisloff, U. 2013. Aerobic capacity reference data in 3816 healthy men and women 20-90 years. PloS One 8 (5), e64319.

Löllgen, H., Bockenhoff, A. & Knapp, G. 2009. Physical activity and all-cause mortality: an updated meta-analysis with different intensity categories. International Journal of Sports Medicine 30 (3), 213-224.

Lopez-Lluch, G., Irusta, P. M., Navas, P. & de Cabo, R. 2008. Mitochondrial biogenesis and healthy aging. Experimental Gerontology 43 (9), 813-819.

McArdle, W., Katch, F. & Katch, V. 2015. Exercise Physiology: Nutriton, Energy and Human Performance. Wolters Kluwer Health.

McClintock, B. 1941. The Stability of Broken Ends of Chromosomes in Zea Mays. Genetics 26 (2), 234-282.

Mehlen, P., Kretz-Remy, C., Preville, X. & Arrigo, A. P. 1996. Human hsp27, Drosophila hsp27 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFalpha-induced cell death. The EMBO journal 15 (11), 2695-2706.

Metter, E. J., Talbot, L. A., Schrager, M. & Conwit, R. 2002. Skeletal muscle strength as a predictor of all-cause mortality in healthy men. The Journals of Gerontology (Series A) 57 (10), B359-65.

Michishita, E., McCord, R. A., Berber, E., Kioi, M., Padilla-Nash, H., Damian, M., Cheung, P., Kusumoto, R., Kawahara, T. L., Barrett, J. C., Chang, H. Y., Bohr, V. A., Ried, T., Gozani, O. & Chua, K. F. 2008. SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. Nature 452 (7186), 492-496.

Mier, C. M., Turner, M. J., Ehsani, A. A. & Spina, R. J. 1997. Cardiovascular adaptations to 10 days of cycle exercise. Journal of Applied Physiology 83 (6), 1900-1906.

Mogensen, M., Sahlin, K., Fernstrom, M., Glintborg, D., Vind, B. F., Beck-Nielsen, H. & Hojlund, K. 2007. Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes 56 (6), 1592-1599.

Morris, E. M., Jackman, M. R., Johnson, G. C., Liu, T. W., Lopez, J. L., Kearney, M. L., Fletcher, J. A., Meers, G. M., Koch, L. G., Britton, S. L., Rector, R. S., Ibdah, J. A., MacLean, P. S. & Thyfault, J. P. 2014. Intrinsic aerobic capacity impacts susceptibility to acute high-fat diet-induced hepatic steatosis. American Journal of Physiology 307 (4), E355-64.

Morris, M. G., Dawes, H., Howells, K., Scott, O. M., Cramp, M. & Izadi, H. 2011. Alterations in peripheral muscle contractile characteristics following high and low intensity bouts of exercise. European Journal of Applied Physiology 112(1):337-43.

Muller, H. J. 1938. The remaking of chromosomes Collecting Net. 13, 181-198.

Murlasits, Z., Cutlip, R. G., Geronilla, K. B., Rao, K. M., Wonderlin, W. F. & Alway, S. E. 2006. Resistance training increases heat shock protein levels in skeletal muscle of young and old rats. Experimental Gerontology 41 (4), 398-406.

Myers, J., Prakash, M., Froelicher, V., Do, D., Partington, S. & Atwood, J. E. 2002. Exercise capacity and mortality among men referred for exercise testing. The New England Journal of Medicine 346 (11), 793-801.

Nair, K. S. 2005. Aging muscle. The American Journal of Clinical Nutrition 81 (5), 953-963.

Nakagawa, T. & Guarente, L. 2011. Sirtuins at a glance. Journal of Cell Science 124 (Pt 6), 833-838.

Navarro, A., Gomez, C., Lopez-Cepero, J. M. & Boveris, A. 2004. Beneficial effects of moderate exercise on mice aging: survival, behavior, oxidative stress, and mitochondrial electron transfer. American Journal of Physiology 286 (3), R505-11.

Noland, R. C., Thyfault, J. P., Henes, S. T., Whitfield, B. R., Woodlief, T. L., Evans, J. R., Lust, J. A., Britton, S. L., Koch, L. G., Dudek, R. W., Dohm, G. L.,

Cortright, R. N. & Lust, R. M. 2007. Artificial selection for high-capacity endurance running is protective against high-fat diet-induced insulin resistance. American Journal of Physiology 293 (1), E31-41.

Novak, C. M., Escande, C., Burghardt, P. R., Zhang, M., Barbosa, M. T., Chini, E. N., Britton, S. L., Koch, L. G., Akil, H. & Levine, J. A. 2010. Spontaneous activity, economy of activity, and resistance to diet-induced obesity in rats bred for high intrinsic aerobic capacity. Hormones and Behavior 58 (3), 355-367.

Olovnikov, A. M. 1996. Telomeres, telomerase, and aging: origin of the theory. Experimental Gerontology 31 (4), 443-448.

Olshansky, S. J., Passaro, D. J., Hershow, R. C., Layden, J., Carnes, B. A., Brody, J., Hayflick, L., Butler, R. N., Allison, D. B. & Ludwig, D. S. 2005. A potential decline in life expectancy in the United States in the 21st century. The New England Journal of Medicine 352 (11), 1138-1145.

Ostling, P., Bjork, J. K., Roos-Mattjus, P., Mezger, V. & Sistonen, L. 2007. Heat shock factor 2 (HSF2) contributes to inducible expression of hsp genes through interplay with HSF1. The Journal of Biological Chemistry 282 (10), 7077-7086.

Overmyer, K. A., Evans, C. R., Qi, N. R., Minogue, C. E., Carson, J. J., Chermside-Scabbo, C. J., Koch, L. G., Britton, S. L., Pagliarini, D. J., Coon, J. J. & Burant, C. F. 2015. Maximal oxidative capacity during exercise is associated with skeletal muscle fuel selection and dynamic changes in mitochondrial protein acetylation. Cell Metabolism 21 (3), 468-478.

Palacios, O. M., Carmona, J. J., Michan, S., Chen, K. Y., Manabe, Y., Ward, J. L.,3rd, Goodyear, L. J. & Tong, Q. 2009. Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle. Aging 1 (9), 771-783.

Pansarasa, O., Bertorelli, L., Vecchiet, J., Felzani, G. & Marzatico, F. 1999. Age-dependent changes of antioxidant activities and markers of free radical damage in human skeletal muscle. Free Radical Biology & Medicine 27 (5-6), 617-622.

Perusse, L., Tremblay, A., Leblanc, C. & Bouchard, C. 1989. Genetic and environmental influences on level of habitual physical activity and exercise participation. American Journal of Epidemiology 129 (5), 1012-1022.

Physical Activity Guidelines Advisory Committee 2008. *Physical Activity Guidelines Advisory Committee. Physical Activity Guidelines Advisory Committee Report*. Washington, DC: U.S.Department of Health and Human Services.

Pilegaard, H., Saltin, B. & Neufer, P. D. 2003. Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. The Journal of Physiology 546 (3), 851-858.

Pollock, M. L., Miller, H. S.,Jr & Wilmore, J. 1974. Physiological characteristics of champion American track athletes 40 to 75 years of age. Journal of Gerontology 29 (6), 645-649.

Poole, D. C. & Mathieu-Costello, O. 1996. Relationship between fiber capillarization and mitochondrial volume density in control and trained rat soleus and plantaris muscles. Microcirculation 3 (2), 175-186.

Prelog, M. 2006. Aging of the immune system: a risk factor for autoimmunity? Autoimmunity Reviews 5 (2), 136-139.

Pringle, J. S., Doust, J. H., Carter, H., Tolfrey, K., Campbell, I. T., Sakkas, G. K. & Jones, A. M. 2003. Oxygen uptake kinetics during moderate, heavy and severe intensity "submaximal" exercise in humans: the influence of muscle fibre type and capillarisation. European Journal of Applied Physiology 89 (3-4), 289-300.

Ren, Y. Y., Overmyer, K. A., Qi, N. R., Treutelaar, M. K., Heckenkamp, L., Kalahar, M., Koch, L. G., Britton, S. L., Burant, C. F. & Li, J. Z. 2013. Genetic analysis of a rat model of aerobic capacity and metabolic fitness. PloS one 8 (10), e77588.

Richardson, R. S. 2000. What governs skeletal muscle VO2max? New evidence. Medicine and Science in Sports and Exercise 32 (1), 100-107.

Rijsdijk, F. V. & Sham, P. C. 2002. Analytic approaches to twin data using structural equation models. Briefings in Bioinformatics 3 (2), 119-133.

Rikke, B. A. & Johnson, T. E. 2004. Lower body temperature as a potential mechanism of life extension in homeotherms. Experimental Gerontology 39 (6), 927-930.

Rivas, D. A., Lessard, S. J., Saito, M., Friedhuber, A. M., Koch, L. G., Britton, S. L., Yaspelkis, B. B.,3rd & Hawley, J. A. 2011. Low intrinsic running capacity is associated with reduced skeletal muscle substrate oxidation and lower mitochondrial content in white skeletal muscle. American Journal of Physiology 300 (4), R835-43.

Robinson, S. 1938. Experimental studies of physical fitness in relation in master athletes. Arbeitsphysiologie 10, 251-323.

Robinson, D. M., Ogilvie, R. W., Tullson, P. C. & Terjung, R. L. 1994. Increased peak oxygen consumption of trained muscle requires increased electron flux capacity. Journal of Applied Physiology 77 (4), 1941-1952.

Rogina, B. & Helfand, S. L. 2004. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. Proceedings of the National Academy of Sciences of the United States of America 101 (45), 15998-16003.

Rolfe, D. F., Newman, J. M., Buckingham, J. A., Clark, M. G. & Brand, M. D. 1999. Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver and to SMR. The American Journal of Physiology 276 (3 Pt 1), C692-9.

Rose, G., Dato, S., Altomare, K., Bellizzi, D., Garasto, S., Greco, V., Passarino, G., Feraco, E., Mari, V., Barbi, C., BonaFe, M., Franceschi, C., Tan, Q., Boiko, S., Yashin, A. I. & De Benedictis, G. 2003. Variability of the SIRT3 gene, human silent information regulator Sir2 homologue, and survivorship in the elderly. Experimental Gerontology 38 (10), 1065-1070.

Rosen, M. J., Sorkin, J. D., Goldberg, A. P., Hagberg, J. M. & Katzel, L. I. 1998. Predictors of age-associated decline in maximal aerobic capacity: a comparison of four statistical models. Journal of Applied Physiology 84 (6), 2163-2170.

Roth, G. S., Lane, M. A., Ingram, D. K., Mattison, J. A., Elahi, D., Tobin, J. D., Muller, D. & Metter, E. J. 2002. Biomarkers of caloric restriction may predict longevity in humans. Science 297 (5582), 811.

Rottensteiner, M., Leskinen, T., Niskanen, E., Aaltonen, S., Mutikainen, S., Wikgren, J., Heikkila, K., Kovanen, V., Kainulainen, H., Kaprio, J., Tarkka, I. M. & Kujala, U. M. 2015. Physical activity, fitness, glucose homeostasis, and brain morphology in twins. Medicine and Science in Sports and Exercise 47 (3), 509-518.

Rousset, S., Alves-Guerra, M. C., Mozo, J., Miroux, B., Cassard-Doulcier, A. M., Bouillaud, F. & Ricquier, D. 2004. The biology of mitochondrial uncoupling proteins. Diabetes 53 (Suppl 1), S130-135.

Ruiz, J. R., Moran, M., Arenas, J. & Lucia, A. 2011. Strenuous endurance exercise improves life expectancy: it's in our genes. British Journal of Sports Medicine 45 (3), 159-161.

Saanijoki, T., Nummenmaa, L., Eskelinen, J. J., Savolainen, A. M., Vahlberg, T., Kalliokoski, K. K. & Hannukainen, J. C. 2015. Affective Responses to Repeated Sessions of High-Intensity Interval Training. Medicine and Science in Sports and Exercise 47 (12), 2604-2611.

Sack, M. N. & Finkel, T. 2012. Mitochondrial metabolism, sirtuins, and aging. Cold Spring Harbor perspectives in biology 1; 4(12)

Saigal, S. & Bhargava, A. 2012. Progeria: pathogenesis and oral manifestation--a review. Kathmandu University Medical Journal 10 (37), 72-76.

Samitz, G., Egger, M. & Zwahlen, M. 2011. Domains of physical activity and all-cause mortality: systematic review and dose-response meta-analysis of cohort studies. International Journal of Epidemiology 40 (5), 1382-1400.

Sanchez-Alavez, M., Alboni, S. & Conti, B. 2011. Sex- and age-specific differences in core body temperature of C57Bl/6 mice. Age 33 (1), 89-99.

Sarna, S., Kaprio, J., Sistonen, P. & Koskenvuo, M. 1978. Diagnosis of twin zygosity by mailed questionnaire. Human Heredity 28 (4), 241-254.

Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G. & Garrido, C. 2007. Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. Journal of Leukocyte Biology 81 (1), 15-27.

Schnohr, P., O'Keefe, J. H., Marott, J. L., Lange, P. & Jensen, G. B. 2015. Dose of jogging and long-term mortality: the Copenhagen City Heart Study. Journal of the American College of Cardiology 65 (5), 411-419.

Schoenmaker, M., de Craen, A. J., de Meijer, P. H., Beekman, M., Blauw, G. J., Slagboom, P. E. & Westendorp, R. G. 2006. Evidence of genetic enrichment for exceptional survival using a family approach: the Leiden Longevity Study. European Journal of Human Genetics 14 (1), 79-84.

Shephard, R. J. 1966. World standards of cardiorespiratory performance. Archives of Environmental Health 13 (5), 664-672.

Silvennoinen, M., Rantalainen, T. & Kainulainen, H. 2014. Validation of a method to measure total spontaneous physical activity of sedentary and voluntary running mice. Journal of Neuroscience Methods 235 (0), 51-58.

Skytthe, A., Pedersen, N. L., Kaprio, J., Stazi, M. A., Hjelmborg, J. V., Iachine, I., Vaupel, J. W. & Christensen, K. 2003. Longevity studies in GenomEUtwin. Twin Research 6 (5), 448-454.

Slonaker & J. R. 1912. The normal activity of the albino rat from birth to natural death, its rate of growth and the duration of life. Journal of Animal Behavior 2, 20-42.

Sohal, R. S. & Dubey, A. 1994. Mitochondrial oxidative damage, hydrogen peroxide release, and aging. Free Radical biology & Medicine 16 (5), 621-626.

Sohal, R. S., Mockett, R. J. & Orr, W. C. 2002. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. Free Radical Biology & Medicine 33 (5), 575-586.

Son, C., Hosoda, K., Ishihara, K., Bevilacqua, L., Masuzaki, H., Fushiki, T., Harper, M. E. & Nakao, K. 2004. Reduction of diet-induced obesity in transgenic mice overexpressing uncoupling protein 3 in skeletal muscle. Diabetologia 47 (1), 47-54.

Speakman, J. R., Talbot, D. A., Selman, C., Snart, S., McLaren, J. S., Redman, P., Krol, E., Jackson, D. M., Johnson, M. S. & Brand, M. D. 2004. Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. Aging Cell 3 (3), 87-95.

Spina, R. J., Chi, M. M., Hopkins, M. G., Nemeth, P. M., Lowry, O. H. & Holloszy, J. O. 1996. Mitochondrial enzymes increase in muscle in response to 7-10 days of cycle exercise. Journal of Applied Physiology 80 (6), 2250-2254.

Stubbe, J. H., Boomsma, D. I., Vink, J. M., Cornes, B. K., Martin, N. G., Skytthe, A., Kyvik, K. O., Rose, R. J., Kujala, U. M., Kaprio, J., Harris, J. R., Pedersen, N. L., Hunkin, J., Spector, T. D. & de Geus, E. J. 2006. Genetic influences on exercise participation in 37,051 twin pairs from seven countries. PloS One 1, e22.

Szczesny, B., Tann, A. W. & Mitra, S. 2010. Age- and tissue-specific changes in mitochondrial and nuclear DNA base excision repair activity in mice: Susceptibility of skeletal muscles to oxidative injury. Mechanisms of Ageing and Development 131 (5), 330-337.

Talbot, L. A., Metter, E. J. & Fleg, J. L. 2000. Leisure-time physical activities and their relationship to cardiorespiratory fitness in healthy men and women 18-95 years old. Medicine and Science in Sports and Exercise 32 (2), 417-425.

Tan, Q., Christiansen, L., von Bornemann Hjelmborg, J. & Christensen, K. 2015. Twin methodology in epigenetic studies. The Journal of Experimental Biology 218 (1), 134-139.

Termin, A., Staron, R. S. & Pette, D. 1989. Myosin heavy chain isoforms in histochemically defined fiber types of rat muscle. Histochemistry 92 (6), 453-457.

Thyfault, J. P., Rector, R. S., Uptergrove, G. M., Borengasser, S. J., Morris, E. M., Wei, Y., Laye, M. J., Burant, C. F., Qi, N. R., Ridenhour, S. E., Koch, L. G., Britton, S. L. & Ibdah, J. A. 2009. Rats selectively bred for low aerobic capacity have reduced hepatic mitochondrial oxidative capacity and susceptibility to hepatic steatosis and injury. The Journal of Physiology 587 (8), 1805-1816.

Timmons, J. A., Knudsen, S., Rankinen, T., Koch, L. G., Sarzynski, M., Jensen, T., Keller, P., Scheele, C., Vollaard, N. B., Nielsen, S., Akerstrom, T., MacDougald, O. A., Jansson, E., Greenhaff, P. L., Tarnopolsky, M. A., van Loon, L. J., Pedersen, B. K., Sundberg, C. J., Wahlestedt, C., Britton, S. L. & Bouchard, C. 2010. Using molecular classification to predict gains in maximal aerobic capacity following endurance exercise training in humans. Journal of Applied Physiology 108 (6), 1487-1496.

Titchenal, C. A. 1988. Exercise and food intake. What is the relationship? Sports Medicine 6 (3), 135-145.

Toth, M. J., Gardner, A. W., Ades, P. A. & Poehlman, E. T. 1994. Contribution of body composition and physical activity to age-related decline in peak VO2 in men and women. Journal of Applied Physiology 77 (2), 647-652.

Trappe, S. W., Costill, D. L., Vukovich, M. D., Jones, J. & Melham, T. 1996. Aging among elite distance runners: a 22-yr longitudinal study. Journal of Applied Physiology 80 (1), 285-290.

Trayhurn, P., Thurlby, P. L. & James, W. P. 1977. Thermogenic defect in pre-obese ob/ob mice. Nature 266 (5597), 60-62.

Trifunovic, A. & Larsson, N. G. 2008. Mitochondrial dysfunction as a cause of ageing. Journal of Internal Medicine 263 (2), 167-178.

van den Berg, S. A., van Marken Lichtenbelt, W., Willems van Dijk, K. & Schrauwen, P. 2011. Skeletal muscle mitochondrial uncoupling, adaptive thermogenesis and energy expenditure. Current Opinion in Clinical Nutrition and Metabolic Care 14 (3), 243-249.

van Dongen, J., Slagboom, P. E., Draisma, H. H., Martin, N. G. & Boomsma, D. I. 2012. The continuing value of twin studies in the omics era. Nature Reviews. Genetics 13 (9), 640-653.

Van Remmen, H. & Richardson, A. 2001. Oxidative damage to mitochondria and aging. Experimental Gerontology 36 (7), 957-968.

Vieira-Potter, V. J., Padilla, J., Park, Y. M., Welly, R. J., Scroggins, R. J., Britton, S. L., Koch, L. G., Jenkins, N. T., Crissey, J. M., Zidon, T., Morris, E. M., Meers, G. M. & Thyfault, J. P. 2015. Female rats selectively bred for high intrinsic aerobic fitness are protected from ovariectomy-associated metabolic dysfunction. American Journal of Physiology 308 (6), R530-42.

Virtanen, K. A., Lidell, M. E., Orava, J., Heglind, M., Westergren, R., Niemi, T., Taittonen, M., Laine, J., Savisto, N. J., Enerback, S. & Nuutila, P. 2009. Functional brown adipose tissue in healthy adults. The New England Journal of Medicine 360 (15), 1518-1525.

Waalen, J. & Buxbaum, J. N. 2011. Is older colder or colder older? The association of age with body temperature in 18,630 individuals. The Journals of Gerontology (Series A) 66 (5), 487-492.

Waller, K., Kaprio, J. & Kujala, U. M. 2008. Associations between long-term physical activity, waist circumference and weight gain: a 30-year longitudinal twin study. International Journal of Obesity 32 (2), 353-361.

Walsh, B., Hooks, R. B., Hornyak, J. E., Koch, L. G., Britton, S. L. & Hogan, M. C. 2006. Enhanced mitochondrial sensitivity to creatine in rats bred for high aerobic capacity. Journal of Applied Physiology 100 (6), 1765-1769.

Wang, Y., Michikawa, Y., Mallidis, C., Bai, Y., Woodhouse, L., Yarasheski, K. E., Miller, C. A., Askanas, V., Engel, W. K., Bhasin, S. & Attardi, G. 2001. Muscle-specific mutations accumulate with aging in critical human mtDNA control sites for replication. Proceedings of the National Academy of Sciences of the United States of America 98 (7), 4022-4027.

Wei, Y. H. & Lee, H. C. 2002. Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. Experimental Biology and Medicine 227 (9), 671-682.

Wenger, H. A. & Bell, G. J. 1986. The interactions of intensity, frequency and duration of exercise training in altering cardiorespiratory fitness. Sports Medicine 3 (5), 346-356.

Westerblad, H., Allen, D. G., Bruton, J. D., Andrade, F. H. & Lannergren, J. 1998. Mechanisms underlying the reduction of isometric force in skeletal muscle fatigue. Acta Physiologica Scandinavica 162 (3), 253-260.

Westerblad, H., Lee, J. A., Lannergren, J. & Allen, D. G. 1991. Cellular mechanisms of fatigue in skeletal muscle. The American Journal of Physiology 261 (2 Pt 1), C195-209.

Westerheide, S. D., Anckar, J., Stevens, S. M.,Jr, Sistonen, L. & Morimoto, R. I. 2009. Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. Science 323 (5917), 1063-1066.

Wilhite, D. P., Mickleborough, T. D., Laymon, A. S. & Chapman, R. F. 2013. Increases in .VO2max with "live high-train low" altitude training: role of ventilatory acclimatization. European Journal of Applied Physiology 113 (2), 419-426.

Williams, P. T. 2001. Physical fitness and activity as separate heart disease risk factors: a meta-analysis. Medicine and Science in Sports and Exercise 33 (5), 754-761.

Wisloff, U., Najjar, S. M., Ellingsen, O., Haram, P. M., Swoap, S., Al-Share, Q., Fernstrom, M., Rezaei, K., Lee, S. J., Koch, L. G. & Britton, S. L. 2005. Cardiovascular risk factors emerge after artificial selection for low aerobic capacity. Science 307 (5708), 418-420.

Yamashita, H., Yamamoto, M., Ookawara, T., Sato, Y., Ueno, N. & Ohno, H. 1994. Discordance between thermogenic activity and expression of uncoupling protein in brown adipose tissue of old rats. Journal of Gerontology 49 (2), B54-59.

ORIGINAL PAPERS

I

RATS BRED FOR LOW AEROBIC CAPACITY BECOME PROMPTLY FATIGUED AND HAVE SLOW METABOLIC RECOVERY AFTER STIMULATED, MAXIMAL MUSCLE CONTRACTIONS

by

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Rats Bred for Low Aerobic Capacity Become Promptly Fatigued and Have Slow Metabolic Recovery after Stimulated, Maximal Muscle Contractions

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Abstract

AIM: Muscular fatigue is a complex phenomenon affected by muscle fiber type and several metabolic and ionic changes within myocytes. Mitochondria are the main determinants of muscle oxidative capacity which is also one determinant of muscle fatigability. By measuring the concentrations of intracellular stores of high-energy phosphates it is possible to estimate the energy production efficiency and metabolic recovery of the muscle. Low intrinsic aerobic capacity is known to be associated with reduced mitochondrial function. Whether low intrinsic aerobic capacity also results in slower metabolic recovery of skeletal muscle is not known. Here we studied the influence of intrinsic aerobic capacity on in vivo muscle metabolism during maximal, fatiguing electrical stimulation.

METHODS: Animal subjects were genetically heterogeneous rats selectively bred to differ for non–trained treadmill running endurance, low capacity runners (LCRs) and high capacity runners (HCRs) (n = 15–19). We measured the concentrations of major phosphorus compounds and force parameters in a contracting triceps surae muscle complex using ^{31}P-Magnetic resonance spectroscopy (^{31}P-MRS) combined with muscle force measurement from repeated isometric twitches.

RESULTS: Our results demonstrated that phosphocreatine re-synthesis after maximal muscle stimulation was significantly slower in LCRs ($p<0.05$). LCR rats also became promptly fatigued and maintained the intramuscular pH poorly compared to HCRs. Half relaxation time (HRT) of the triceps surae was significantly longer in LCRs throughout the stimulation protocol ($p\leq0.05$) and maximal rate of torque development (MRTD) was significantly lower in LCRs compared to HCRs from 2 min 30 s onwards ($p\leq0.05$).

CONCLUSION: We observed that LCRs are more sensitive to fatigue and have slower metabolic recovery compared to HCRs after maximal muscle contractions. These new findings are associated with reduced running capacity and with previously found lower mitochondrial content, increased body mass and higher complex disease risk of LCRs.

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Introduction

Muscular fatigue is characterized with decline of force production caused by prolonged activation of skeletal muscle. It is known that muscle fiber types, characterized by differences in myosin heavy chain (MHC) composition, vary in force-generating capacity and resistance to fatigue [1–4]. Hence muscle fiber type composition is one key factor affecting the sensitivity of muscle to fatigue. At a myocellular level the reduced force can be caused by various metabolic or ionic changes, such as reduced pH, accumulation of inorganic phosphate (Pi) into myocytes, decreased intracellular calcium ion (Ca^{2+}) release or reduced sensitivity of the myofilaments to Ca^{2+}-ions [5,6]. These changes in myocellular metabolites during muscle fatigue are often linked to each other. For example reduced pH and accumulation of Pi lead to reduced Ca^{2+} sensitivity and reduced maximum tension. This in turn has an important contribution to the force decline, especially with repeated maximal muscle stimulation [7].

In addition to muscle fiber type composition and myocellular metabolic and ionic changes the capacity to resist fatigue is determined by muscle oxidative capacity [8–10]. Muscle oxidative capacity in turn is mainly determined by the amount and quality

of myocellular mitochondria. These parameters vary between individuals due to differences in genotype and in the amount of physical activity [11–14]. Besides being a major contributor to muscle oxidative capacity, mitochondria have a crucial role in integrating the key metabolic fluxes in the cell. Phosphocreatine (PCr) and creatine (Cr) provide an intracellular, high-energy phosphate buffering system, essential to maintain adenosine-triphosphate (ATP) levels in tissues with high energy demands. Internal stores of high-energy phosphates provide additional ATP at the onset of exercise in skeletal muscle.

With ^{31}P-Magnetic resonance spectroscopy (^{31}P-MRS) it is possible to measure the concentrations of the major phosphorus compounds in a contracting muscle, *i.e.*, PCr and Pi [15,16]. With these parameters one can estimate the energy production efficiency of the muscle, such as the speed of PCr degradation and resynthesis, provided that the simultaneous force output is recorded [17]. Until recently, this method required invasive systems to induce muscle contraction electrically, which in turn necessitated the sacrifice of the animal after the experiment. Giannesini and colleagues (2005) designed and constructed a new experimental setup for noninvasive MRS investigation of muscle function in contracting rat triceps surae muscle complex (gastrocnemius, soleus and plantaris) [18]. This maximal intermittent isometric fatigue protocol provides a reliable way to study the development of muscle fatigue of the whole muscle without occlusion of the blood flow. Due to the noninvasive approach, muscle metabolic properties can be followed through an individual's life-span, which enables the evaluation of inherited muscle properties together with effects of environmental factors (*e.g.* exercise training) with aging.

Recent studies reveal that low aerobic capacity is associated with various metabolic and morphological properties in skeletal muscle, which in turn have pronounced effects on whole body level in health and disease [19–23]. Since impaired oxidative metabolism underlies several complex diseases, such as type 2 diabetes and cardiovascular disease [24,25], it is generally thought that the ability to increase oxygen transport and utilization capacity by exercise training stimulus helps to prevent or ameliorate developing complex disease. The level of exercise capacity, however, differs substantially between individuals. The primary assumption is that this heterogeneity is a consequence of variation distributed across a large number of genes for both the intrinsic (untrained) aerobic capacity and that accrued from exercise training. To address the intrinsic component, Koch and Britton developed a contrasting animal model system by selectively breeding rats for low and high endurance treadmill running capacity in the untrained condition [10]. These genetically heterogeneous and widely segregated rat strains, termed low capacity runner (LCR) and high capacity runner (HCR), are well suited divergent models for evaluating the interplay of genetic and environmental factors as determinants of health and disease.

Since intrinsic aerobic capacity has pronounced effects in health, complex disease risk and life expectancy [19,22,26] the differences on whole body level of HCR/LCR rats should also be observable in tissue level. In this study the focus is on skeletal muscle. By strict measurement criteria, LCRs fatigue sooner during maximal treadmill running test compared to HCRs [10] but it is not known whether these strains differ in parameters related to maximal muscle stimulation. Therefore, the purpose of this study was to examine the effects of intrinsic aerobic capacity on skeletal muscle metabolism *in vivo* during maximal, fatiguing stimulation in non-trained LCR/HCR rats. We hypothesize that low intrinsic aerobic capacity is associated with fatigue sensitivity, slow metabolic recovery and poor intramuscular pH homeostasis. We also hypothesize, that compared to HCRs, the twitch parameters of LCRs are more typical of fast twitch muscles due to larger relative amount of type 2b muscle fibers [19,27,28].

Materials and Methods

The HCR/LCR contrasting rat model system was produced via two-way artificial selection, starting from a founder population of genetically heterogeneous rats (N:NIH stock) in 1996, as described previously [10]. Endurance running capacity was assessed at the University of Michigan (Ann Arbor, Michigan, USA) with a speed-ramped treadmill running test (15° slope, initial velocity of 10 m min^{-1}, increased 1 m min every 2 min) when the rats where 11 weeks of age (model Exer-4; Columbus Instruments, Columbus, OH). Rats were subsequently shipped to University of Jyväskylä when 16 weeks old. For experiments described here, 34 female HCR and LCR rats (nHCR = 19, nLCR = 15) weighing 194–332 g were used. Rats were from the 23rd generation of selection and 8 months of age when studied. Rats were housed 2/cage in an environmentally controlled facility (12/12 h light-dark cycle, 22°C) and received water and standard feed *ad libitum*.

Ethics Statement

This study was approved by the National Animal Experiment Board, Finland (Permit number ESAVI-2010-07989/Ym-23). The 31P-MRS acquisition protocol was performed under isoflurane anesthesia.

Animal Preparation

The non-invasive MRS investigation setup was modified from the protocol designed by Giannesini et al. [18]. Rats were deprived of food for 2 h before measurements. Basal body temperature and masswere measured at rest before each investigation. Rats were anesthetized in an induction chamber with 4% isoflurane mixed in 30% O_2 and 70% N_2O. The right lower hindlimb was shaved and conductive electrolyte gel was applied at the heel and knee levels to optimize electrical stimulation of triceps surae muscle complex. Each rat was placed in a home-built cradle designed for non-invasive functional investigation of the right triceps surae. The cradle had a built-in strain-gauge sensor (HBM, 1-LY41-6/1000, Darmstadt, Germany) and two transcutaneous electrodes to elicit and measure maximal twitch responses under isometric conditions. The foot was positioned on the pedal and the lower hind limb was centered on a flat radio frequency (RF) coil used for MRS measurement (Fig. 1). The pedal was held constant at a 30 degree angle. Supramaximal square-wave pulses (1-ms duration, current range: 10–19 mA, altered to maximize twitch force) were delivered transcutaneously with a constant current stimulator (Digitimer Stimulator DS7, Digitimer Ltd., Hertfordshire, U.K.) to obtain the maximal isometric twitch responses. Throughout the experiment, anesthesia was maintained by a gas inhalation through a facemask continuously supplied with 1.2–1.75% isoflurane in 30% O_2 and 70% N_2O. Corneas were protected from drying by application of ophthalmic cream (Viscotears, Novartis Pharmaceuticals, U.K.). The facemask was connected to open-circuit gas anesthesia equipment. During anesthesia, animal body temperature was maintained with a heated pad.

31P-MRS Acquisition and Data Processing

Investigations were performed in a 4.7 T horizontal magnet (Magnex Scientific, Abdington, U.K.). A flat surface transmit/receive RF coil (26-mm-diameter ^{1}H- and 20-mm-diameter ^{31}P-coil) was used. The triceps surae muscle complex was carefully placed in the center of the coil combination, and the volume on

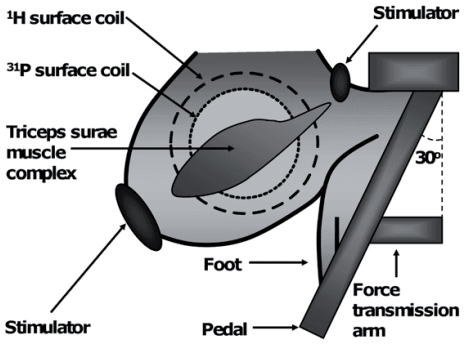

Figure 1. Schematic representation of the setup for measuring rat triceps surae muscle complex function with MR investigation. Triceps surae contractions were induced indirectly via electrical stimulation at the heel and knee levels. Muscle performance was measured with a force transducer which was attached to the pedal. Information about the leg position was acquired with a ^{1}H-MR surface coil and muscle metabolic functions were studied with ^{31}P-MRS surface coil.
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coil was shimmed using ^{1}H nuclear magnetic resonance (NMR) signal followed by pilot images with gradient echo pulse sequence to ensure that the position of the muscle was correct. ^{31}P-MR spectra from the triceps surae were continuously acquired with pulse-acquire technique using 130 μs long, 90 degree block pulse for excitation, 1.855 s time to repeat, 4 kHz bandwidth covered with 2000 points. Prior to stimulation, a baseline spectrum was collected (256 averages, 8 min) and during the stimulation and recovery, a total of 15 spectra were acquired, resulting in temporal resolution of 56 seconds. Areas of PCr and Pi were obtained by a time-domain fitting routine using the AMARES-MRUI Fortran code [29]. PCr and Pi values were normalized to their initial values. Intramuscular pH was calculated from the chemical shift difference between Pi and PCr [30]. The recovery of PCr level was compared by adjusting a linear trend line between time points 7–9 min and comparing the individual slope values. The chosen time-interval showed rapid linear increase in the analyzed variable.

Stimulation Protocol and Signal Analysis

The stimulation protocol consisted of baseline (8 min), stimulation (6 min) and recovery (8 min) measurements. Repeated isometric muscle twitches were elicited through electrical stimulation of triceps surae muscle complex at a frequency of 3.33 Hz (in total 1200 stimuli). Force signal from the strain-gauge sensor was amplified, converted to digital signals by a 32-bit analog to digital converter (Power 1401, CED Ltd., Cambridge, U.K.), and processed using dedicated software (Signal software, CED Ltd.). From each individual twitch response, 1) maximal force, 2) maximal rate of torque development (MRTD) and 3) half-relaxation time (HRT) were analyzed throughout the stimulation period. The twitch properties were then averaged over 15 second epochs (in total 24 averages of 50 stimuli). In addition, twitch force and MRTD values were normalized with their maximum value during the stimulation to compute relative rate of reduction (% of maximum s^{-1}) during the time-course of transcutaneous electrical stimulation. A linear trend line was adjusted over 100 twitch responses in individual time-intervals that showed rapid linear reduction in the analyzed variables.

Statistical Analyses

All values are expressed as mean ± standard error of the mean (SEM). Statistical analyses for all variables were carried out using SPSS for Windows 13.0 statistical software (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to investigate within group normality for a given parameter of interest. Levene's test was conducted to assess the homogeneity of the variance assumption. When assumptions were met independent-samples t-test was used. A statistical comparison of body mass and mean distance run to exhaustion was made using independent-samples t-test. The recovery of PCr level was compared with independent-samples t-test by comparing the individual slope values. Since the normality or equality of variance assumptions were not met, statistical comparisons of other studied parameters between LCR and HCR groups were made using Mann-Whitney test by comparing LCRs and HCRs at every time point. The twitch property values from the first last epochs (Table 1) were also compared using Mann-Whitney test. The rate of reduction in relative twitch force and MRTD (Table 2) were analyzed using Mann-Whitney test by comparing the individual slope values. P-values less than 0.05 were considered statistically significant.

Results

Background Data

LCR rats were 26% heavier compared to HCRs, the mean body mass of LCRs was 289±7 g and for HCRs 229±5 g ($p<0.001$). The mean distance run at exhaustion was 305±4 m for LCRs *versus* 2093±25 m for HCRs ($p<0.001$), representing a 586% difference in running distance.

^{31}P-MRS

PCr. ^{31}P-MRS results demonstrated that PCr resynthesis after stimulation was significantly slower in LCRs when comparing the slope values of linear trend lines adjusted between time points 7–9 min ($p<0.05$) (Fig. 2A). In both rat strains, the decrease of PCr from the resting level ($t = 0$) started right after stimulation. PCr level also began to increase in both strains immediately after the stimulation protocol was over ($t = 6$). LCR rats had significantly lower PCr value in time point 2 min of stimulation compared to HCR rats.

Pi. Pi levels increased similarly in both rat strains during stimulation period and reached the highest level after 4 minutes of stimulation (Fig. 2B). During recovery period Pi decreased and almost reached back to the starting level. There were no significant differences in the levels of Pi between the groups.

Table 1. Comparison of initial and end values of the twitch properties.

Parameter	Epoch	LCR	HCR	P
Twitch force	First	2.87±0.25	2.51±0.13	0.206
(N)	Last	1.01±0.11	1.29±0.10	<0.05
MRTD	First	184±17	167±10	0.477
(N s-1)	Last	75±8	98±9	<0.01
HRT	First	16.5±0.4	14.1±0.3	<0.001
(ms)	Last	15.2±0.4	13.5±0.5	<0.01

Initial values of twitch force, MRTD and HRT from First epoch (0–15 s) and from Last epoch (345–360 s) during the electrical stimulation. Values are expressed as mean ± SEM.
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Table 2. Rate of change in twitch properties.

Parameter	LCR	HCR	P
Slope	−0.19±0.02	−0.13±0.06	<0.05
(Force % of max min-1)			
Slope	−0.17±0.02	−0.12±0.01	<0.05
(MRTD % of max min-1)			

The rates of change in relative (% of maximal) twitch force and MRTD in individual time-intervals (100 twitches) during the electrical stimulation. Values are expressed as mean ± SEM.
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pH. LCRs maintained the intramuscular pH more poorly compared to HCRs (Fig. 2C). LCRs had significantly lower pH values between stimulation periods 3–5 min and the levels remained lower than the corresponding pH levels of HCRs during the whole protocol, except for the time points 0 and 9 min. At the beginning of the stimulation protocol both rat strains had an equivalent pH value of 7.00. At end of the test period, HCRs had on average a pH of 6.78, whereas LCRs had more reduced pH of 6.55.

Twitch Properties

Initial and End Values of the Twitch Properties. Mean twitch force curves from the first epoch (0–15 s) and from the last epoch (345–360 s) are presented in figures 3A and 3B. The corresponding twitch property values are presented in Table 1. During the first epoch, when the effect of fatigue is minimal, LCRs seemed to show higher initial maximal twitch force, although this was not statistically significant. However, the initial MRDT did not differ between LCRs and HCRs. As the effect of fatigue LCRs showed lower maximal twitch force and MRTD compared to HCRs during the last epoch ($p<0.05$). LCRs also had significantly lower MRTD in the last epoch ($p<0.01$) and higher HRT values both in first and last epoch ($p<0.01$) compared to HCRs.

Twitch Properties during Electrical Stimulation. LCRs tended to show higher maximal twitch force than HCRs during the first epochs (0–30 s), although this trend was not significant. Later during the stimulation, LCRs showed a significantly lower twitch force from 2 min 45 s onwards compared to HCRs ($p\leq0.05$) (Fig. 4A). Similarly, LCRs had tendency for higher MRTD ($N\ s^{-1}$) in the beginning of the stimulation protocol than HCRs (Fig. 4B), but this difference did not reach significance. However, the MRTD was significantly lower in LCRs compared to HCRs from 2 min 30 s onwards ($p\leq0.05$). The half relaxation time (Fig. 4C) of the triceps surae was significantly longer in LCRs throughout the stimulation protocol ($p\leq0.05$).

Rate of Change in Twitch Properties. The rate of reduction in the normalized twitch force and MRTD during transcutaneous electrical stimulation occurred more rapidly in LCRs compared to HCRs ($p<0.05$) when the slope of individual twitch responses were compared (Table 2).

Discussion

In the present study we examined noninvasively the characteristics of triceps surae muscle complex metabolism in two genetically heterogeneous rat strains (HCR/LCR) that widely differ for intrinsic (i.e. non-trained) aerobic capacity. Consistent with a lower maximal treadmill running capacity, we found that the skeletal muscle in LCRs become promptly fatigued during maximal muscle stimulation *in vivo*. LCRs have also slower metabolic recovery after continued contractions compared to HCRs.

We hypothesized, that LCRs would have slow metabolic recovery, which is one sign of poor mitochondrial function and/

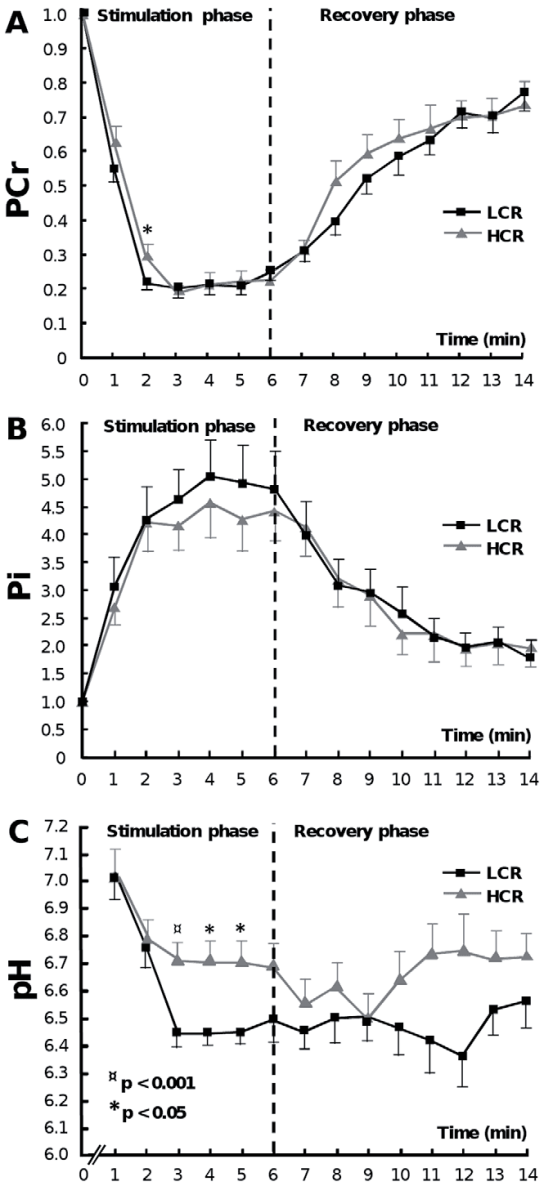

Figure 2. PCr, Pi and pH levels during ^{31}P-MRS acquisition protocol. PCr (A), Pi (B) and pH (C) levels in triceps surae muscle complex during stimulation (6 min) and recovery (8 min) measurements. PCr resynthesis was significantly slower in LCRs compared to HCRs ($p<0.05$) when comparing the individual slope values. LCRs also had significantly lower PCr level in time point 2 min ($p<0.05$). There were no statistical differences between HCR and LCR groups in Pi levels. Intramuscular pH was lower in LCRs throughout the protocol except for the time points 1 and 9 min. Values are expressed as mean ± SEM.
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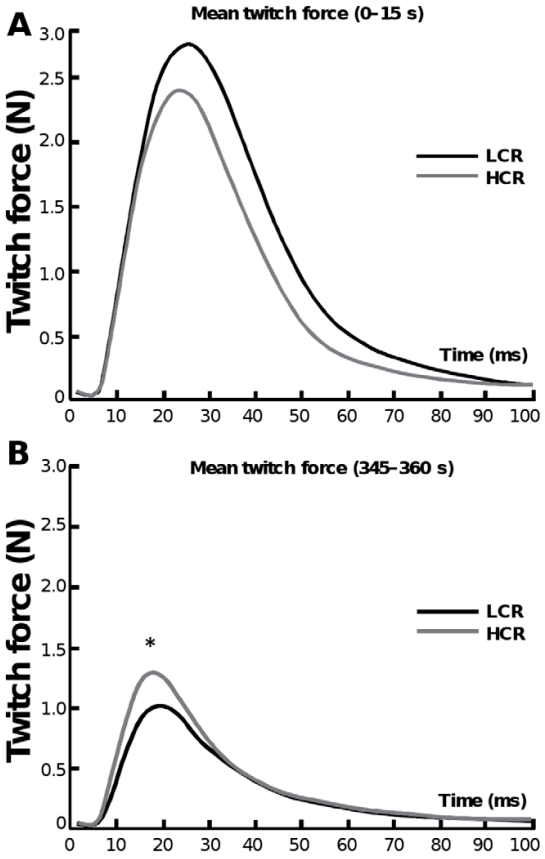

Figure 3. Initial and end values of the twitch properties. The mean twitch force curves from initial epoch (0–15 s) (A) and from last epoch (345–360 s) (B) during electrical stimulation. The corresponding twitch property values are presented in Table 1. LCRs seemed to have slightly higher initial maximal twitch force and MRTD (n.s.). At the end of the stimulation protocol LCRs showed significantly lower maximal twitch force and MRTD compared to LCRs ($p<0.05$). LCRs had significantly higher HRT values both in first and last epoch ($p<0.01$) than HCRs.
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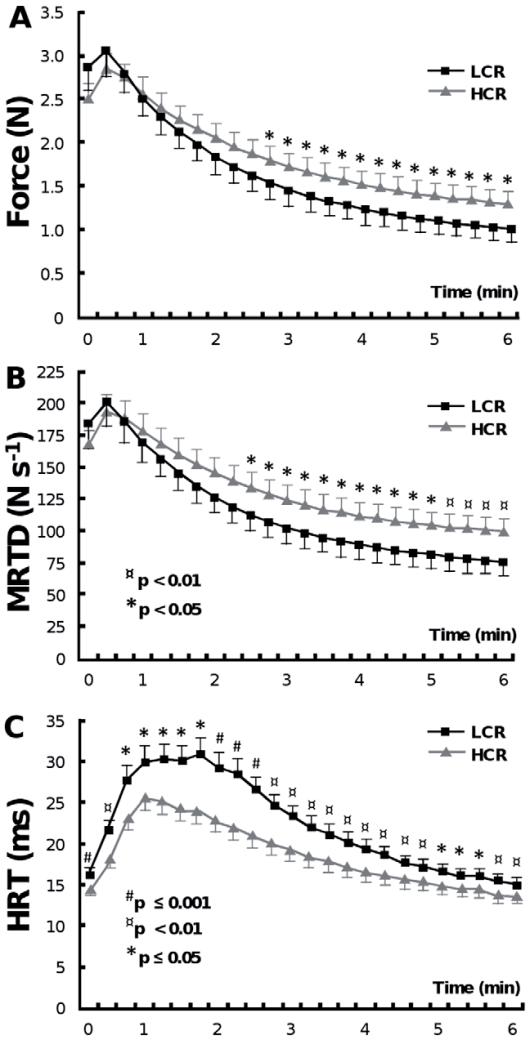

Figure 4. Twitch properties during electrical stimulation. Force (A), MRTD (B) and HRT (C) measured simultaneously with MRS acquisition during 6 min stimulation protocol. From 2 min 45 s onwards LCRs had significantly lower twitch force compared to HCRs ($p\leq0.05$). The MRTD values were significantly lower in LCRs compared to HCRs from 2 min 30 s onwards ($p\leq0.05$). LCRs had higher HRT than HCRs throughout the stimulation protocol ($p\leq0.05$). Values are expressed as mean ± SEM.
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or capacity [30,31]. In previous studies we have shown, that LCRs have poor capillarization and less subsarcolemmal and inter-myofibrillar mitochondria in skeletal muscle compared to HCRs [19]. Rivas *et al* in turn found that the most significant differences in mitochondrial content between these rat strains were in white muscle type [32]. It is likely that the slower PCr recovery of LCRs is partly due to lower mitochondrial content of the muscle compared to HCRs. Diminished oxidative capacity of the mitochondria may also play a role. Indeed, it has been shown that despite similar mitochondrial density, LCRs have reduced mitochondrial respiratory capacity in skeletal muscle compared to HCRs [33]. This is consistent with the finding, that skeletal muscle from obese subjects or from type 2 diabetic patients show reduced mitochondrial oxidative capacity [20,23,25,34].

A novel finding from the present study was that LCRs become promptly fatigued during maximal isometric muscle stimulation (Fig. 4 and Table 2), not only during an aerobic running test. In the present study we used protocol with intermittent isometric twitches used by Giannesini et al [18]. Isometric muscle stimulation method enabled us to hold the muscle still during the whole stimulation period, which is vital to ^{31}P-MRS investigation when using surface coils. Another advantage of the isometric fatigue protocol is the constant muscle length that ensures measured force parameters are not affected by change in the muscle length. The non-invasive muscle stimulation method has been proven to stimulate solely the triceps surae muscle complex and not the antagonist muscles. In fact, Giannesini et al (2005) found in their study, using T_2-weighted MR images, that

the transcutaneous stimulation system activates specifically the gastrocnemius muscle of triceps surae [18]. Since our stimulation system is similar to that used in Giannesini's studies we assume, that also our transcutaneous stimulation mainly stimulates gastrocnemius muscle.

Muscle fibers vary in force-generating capacity, contraction speed and resistance to fatigue [2,3]. Previous analysis of MHC composition revealed that LCRs express more 2b MHC isoform compared to HCRs, where 2a/x MHC was more pronounced [19]. MHC types define the muscle fiber type, which in turn belong to motor units having distinct functional properties: type 2a fibers correspond to fast-contracting fatigue-resistant motor units and type 2b fibers to fast-contracting fatigue-sensitive motor units [4]. Howlett *et al* also showed that LCRs have significantly higher activation of the glycolytic enzyme phosphofructokinase [35]. Our results from force measurement support these findings. During the maximal stimulation LCRs fatigued at a faster rate, whereas HCRs demonstrated fatigue resistance (Table 2). MRTD is a muscle twitch parameter that illustrates how fast the maximal torque level is reached. Muscles that consist mainly of fast-twitch fibers tend to have higher MRTD values. Although LCRs start with a higher twitch force and MRTD at the beginning of the stimulation (0–15 s) the opposite occurs upon reaching the end of the stimulation protocol (345–360 s) (Fig. 4). This supports our hypothesis, that LCRs have greater reliance on fast-contracting and fatiguing muscle fibers compared to HCRs.

It has been shown that HCR and LCR rats have similar resting muscle phosphocreatine, glycogen and ATP contents [35]. LCRs, however, have diminished sensitivity for Cr-induced stimulation of submaximally ADP-stimulated respiration in skeletal muscle compared HCRs [33]. This tendency was also true in the case of non-ADP-stimulated and maximally ADP-stimulated respiration. A diminished sensitivity in skeletal muscle may contribute to the performance differences between LCR and HCR rats, as a consequence of an increased insensitivity of the mitochondrion, and thus the whole muscle, to ADP during exercise. The work of Walsh *et al* demonstrated that HCRs maintain a higher degree of functional coupling between creatine kinase and adenine nucleotide translocase compared to the LCRs in slow-twitch muscle type [33]. Whether these qualitative changes in mitochondrial function are related to inherent muscle oxidative capacity or are directly in response to exercise capacity remains unclear.

In skeletal muscle, the fatiguing stimulation resulted in depletion of PCr, accumulation of Pi, and a decrease in pH. As we hypothesized, LCRs had poor intramuscular pH homeostasis compared to HCRs. The larger contractile-mediated decrease in pH in the triceps surae of LCRs was likely related to the larger glycolytic capacity in fast-twitch muscle fibers (type 2b) of LCRs when compared to HCRs [19,36,37]. More oxidative muscle fibers (type 2a/x) in turn are able to exploit hydrogen ions in the reformation of pyruvic acid and thus maintain a more optimal pH for enzymatic activity longer. In addition to the reduced pH, accumulation of Pi into myocytes has been proposed to play a key role in fatigue development, defined as a decline in force production and slowing of relaxation during prolonged muscle activity [1,5,38]. Giannesini *et al* [39] tested five different fatiguing muscle stimulation protocols with the outcome that Pi and its diprotonated form ($H_2PO_4^-$) affect force production only at the end of the stimulation period. This suggested an indirect, time-dependent effect of these parameters on force production, which might be mediated by an alteration of Ca^{2+} fluxes throughout the sarcoplasmic reticulum (SR) [39]. In fact, it has been shown, that the decline in Ca^{2+} release from SR occurs later in muscle fibers with high capacity for oxidative metabolism [40,41]. Thus, HCRs may have more efficient hydrogen ion buffering and delayed decline in Ca^{2+} release due to higher oxidative capacity in skeletal muscle [19,32], which might help resist fatigue.

We hypothesized, that LCRs might have higher maximal twitch force (N) compared to HCRs due to bigger muscle size and larger relative amount of 2b muscle fibers with better force-generating capacity compared to 2a fibers of HCRs [19,27,28]. No significant difference, however, was observed in maximal twitch force between the groups in the beginning of the stimulation protocol. Interestingly, LCRs had significantly longer half-relaxation times compared to HCRs throughout the study protocol (Fig. 4C). This suggests that LCRs have relatively more slow-twitch (type 1) muscle fibers than HCRs. This result is inconsistent with the observation that, in gastrocnemius muscle, LCRs have slightly less oxidative type fibers compared to HCRs [1]. The differences of twitch time parameters of LCR and HCR rats have several possible explanations. The effect of body and muscle size might be again of importance. Bigger muscles and perhaps longer muscle fibers in LCRs could explain the longer relaxation time [42]. Fatigue, due to either Pi, $H_2PO_4^-$ or Ca^{2+} fluxes, can also increase the half- relaxation time [17].

The possible link between muscle oxidative capacity and metabolic diseases is an interesting topic. We have already shown that low aerobic capacity is related to higher complex disease risk [19]. Our present results suggest that muscle oxidative capacity, muscle fatigue and complex disease risk are connected to each other. Nevertheless, what is the cause and what is the consequence remains unclear. In addition to the study of complex metabolic diseases, there is a growing interest in understanding the connection between aerobic capacity and exercise training in as it relates to longevity. Several large-scale clinical studies have shown that low exercise capacity is the strongest predictor of all-cause mortality [43–45]. It has been demonstrated, that in addition to a variety of biochemical and physiological differences, LCRs have significantly shorter lifespan compared to HCRs [26]. Aging is also associated with a decline in the rate of force development, especially during fast contractions [46,47]. Studies in humans have revealed that HRT increases and the MRTD declines in response to aging [30,48]. Our present study revealed that, in the non-trained state, rats with low aerobic capacity became promptly fatigued during maximal muscle stimulation and had slow metabolic recovery after continued contractions in triceps surae muscle complex. These results supported the previous findings of substantial differences in skeletal muscle properties between these rat strains [19,32,33,35]. It will be of interest for future studies to determine if exercise training can retrieve the negative contractile activity of the LCR as a function of age and the noninvasive nature of the ^{31}P-MRS measures of contractile function as reported here will be of substantial experimental value for studying the complexities of aging.

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Author Contributions

Conceived and designed the experiments: ST MS HK HP. Performed the experiments: ST. Analyzed the data: ST HP. Contributed reagents/materials/analysis tools: JN PT OG LK SB HK. Wrote the paper: ST. Was involved in the revision of the manuscript: MS HP JN PT OG LK SB HK.

References

1. Bogdanis GC. (2012) Effects of physical activity and inactivity on muscle fatigue. Frontiers in Physiology 3: 142.
2. Carroll TJ, Riek S, Carson RG. (2002) The sites of neural adaptation induced by resistance training in humans. The Journal of Physiology 544: 641–652.
3. Burke RE, Levine DN, Tsairis P, Zajac FE,3rd. (1973) Physiological types and histochemical profiles in motor units of the cat gastrocnemius. The Journal of Physiology 234: 723–748.
4. Edstrom L, Kugelberg E. (1968) Histochemical composition, distribution of fibres and fatiguability of single motor units. anterior tibial muscle of the rat. Journal of Neurology, Neurosurgery, and Psychiatry 31: 424–433.
5. Westerblad H, Allen DG, Bruton JD, Andrade FH, Lannergren J. (1998) Mechanisms underlying the reduction of isometric force in skeletal muscle fatigue. Acta Physiologica Scandinavica 162: 253–260.
6. Allen DG, Westerblad H, Lee JA, Lannergren J. (1992) Role of excitation-contraction coupling in muscle fatigue. Sports Medicine (Auckland, N.Z.) 13: 116–126.
7. Westerblad H, Lee JA, Lannergren J, Allen DG. (1991) Cellular mechanisms of fatigue in skeletal muscle. The American Journal of Physiology 261: C195–209.
8. Bassett DR,Jr, Howley ET. (2000) Limiting factors for maximum oxygen uptake and determinants of endurance performance. Medicine and Science in Sports and Exercise 32: 70–84.
9. Holloszy JO, Coyle EF. (1984) Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology 56: 831–838.
10. Koch LG, Britton SL. (2001) Artificial selection for intrinsic aerobic endurance running capacity in rats. Physiological Genomics 5: 45–52.
11. Ukropcova B, Sereda O, de Jonge L, Bogacka I, Nguyen T, et al. (2007) Family history of diabetes links impaired substrate switching and reduced mitochondrial content in skeletal muscle. Diabetes 56: 720–727.
12. Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, et al. (2005) Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. The Journal of Clinical Investigation 115: 3587–3593.
13. Fluck M, Hoppeler H. (2003) Molecular basis of skeletal muscle plasticity–from gene to form and function. Reviews of Physiology, Biochemistry and Pharmacology 146: 159–216.
14. Holloszy JO. (1967) Biochemical adaptations in muscle. effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. The Journal of Biological Chemistry 242: 2278–2282.
15. Bendahan D, Giannesini B, Cozzone PJ. (2004) Functional investigations of exercising muscle: A noninvasive magnetic resonance spectroscopy-magnetic resonance imaging approach. Cellular and Molecular Life Sciences : CMLS 61: 1001–1015.
16. Kemp GJ, Radda GK. (1994) Quantitative interpretation of bioenergetic data from 31P and 1H magnetic resonance spectroscopic studies of skeletal muscle: An analytical review. Magnetic Resonance Quarterly 10: 43–63.
17. Giannesini B, Izquierdo M, Le Fur Y, Cozzone PJ, Bendahan D. (2001) In vivo reduction in ATP cost of contraction is not related to fatigue level in stimulated rat gastrocnemius muscle. The Journal of Physiology 536: 905–915.
18. Giannesini B, Izquierdo M, Le Fur Y, Cozzone PJ, Fingerle J, et al. (2005) New experimental setup for studying strictly noninvasively skeletal muscle function in rat using 1H-magnetic resonance (MR) imaging and 31P-MR spectroscopy. Magnetic Resonance in Medicine : Official Journal of the Society of Magnetic Resonance in Medicine/Society of Magnetic Resonance in Medicine 54: 1058–1064.
19. Kivelä R, Silvennoinen M, Lehti M, Rinnankoski-Tuikka R, Purhonen T, et al. (2010) Gene expression centroids that link with low intrinsic aerobic exercise capacity and complex disease risk. The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 24: 4565–4574.
20. Mogensen M, Sahlin K, Fernstrom M, Glintborg D, Vind BF, et al. (2007) Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes 56: 1592–1599.
21. Koch LG, Britton SL. (2005) Divergent selection for aerobic capacity in rats as a model for complex disease. Integrative and Comparative Biology 45: 405–415.
22. Wisloff U, Najjar SM, Ellingsen O, Haram PM, Swoap S, et al. (2005) Cardiovascular risk factors emerge after artificial selection for low aerobic capacity. Science (New York, N.Y.) 307: 418–420.
23. Kelley DE, He J, Menshikova EV, Ritov VB. (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes 51: 2944–2950.
24. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, et al. (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nature Genetics 34: 267–273.
25. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, et al. (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proceedings of the National Academy of Sciences of the United States of America 100: 8466–8471.
26. Koch LG, Kemi OJ, Qi N, Leng SX, Bijma P, et al. (2011) Intrinsic aerobic capacity sets a divide for aging and longevity. Circulation Research.
27. Bottinelli R, Schiaffino S, Reggiani C. (1991) Force-velocity relations and myosin heavy chain isoform compositions of skinned fibres from rat skeletal muscle. The Journal of Physiology 437: 655–672.
28. Galler S, Schmitt TL, Pette D. (1994) Stretch activation, unloaded shortening velocity, and myosin heavy chain isoforms of rat skeletal muscle fibres. The Journal of Physiology 478 Pt 3: 513–521.
29. Vanhamme L, van den Boogaart A, Van Huffel S. (1997) Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. Journal of Magnetic Resonance (San Diego, Calif.: 1997) 129: 35–43.
30. Arnold DL, Matthews PM, Radda GK. (1984) Metabolic recovery after exercise and the assessment of mitochondrial function in vivo in human skeletal muscle by means of 31P NMR. Magnetic Resonance in Medicine : Official Journal of the Society of Magnetic Resonance in Medicine/Society of Magnetic Resonance in Medicine 1: 307–315.
31. Kemp GJ, Taylor DJ, Radda GK. (1993) Control of phosphocreatine resynthesis during recovery from exercise in human skeletal muscle. NMR in Biomedicine 6: 66–72.
32. Rivas DA, Lessard SJ, Saito M, Friedhuber AM, Koch LG, et al. (2011) Low intrinsic running capacity is associated with reduced skeletal muscle substrate oxidation and lower mitochondrial content in white skeletal muscle. American Journal of Physiology.Regulatory, Integrative and Comparative Physiology 300: R835–43.
33. Walsh B, Hooks RB, Hornyak JE, Koch LG, Britton SL, et al. (2006) Enhanced mitochondrial sensitivity to creatine in rats bred for high aerobic capacity. Journal of Applied Physiology (Bethesda, Md.: 1985) 100: 1765–1769.
34. Bakkman L, Fernstrom M, Loogna P, Rooyackers O, Brandt L, et al. (2010) Reduced respiratory capacity in muscle mitochondria of obese subjects. Obesity Facts 3: 371–375.
35. Howlett RA, Gonzalez NC, Wagner HE, Fu Z, Britton SL, et al. (2003) Selected contribution: Skeletal muscle capillarity and enzyme activity in rats selectively bred for running endurance. Journal of Applied Physiology (Bethesda, Md.: 1985) 94: 1682–1688.
36. Termin A, Staron RS, Pette D. (1989) Myosin heavy chain isoforms in histochemically defined fiber types of rat muscle. Histochemistry 92: 453–457.
37. Fuchtbauer EM, Rowlerson AM, Gotz K, Friedrich G, Mabuchi K, et al. (1991) Direct correlation of parvalbumin levels with myosin isoforms and succinate dehydrogenase activity on frozen sections of rodent muscle. The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society 39: 355–361.
38. Weiner MW, Moussavi RS, Baker AJ, Boska MD, Miller RG. (1990) Constant relationships between force, phosphate concentration, and pH in muscles with differential fatigability. Neurology 40: 1888–1893.
39. Giannesini B, Izquierdo M, Confort-Gouny S, Cozzone PJ, Bendahan D. (2001) Time-dependent and indirect effect of inorganic phosphate on force production in rat gastrocnemius exercising muscle determined by 31P-MRS. FEBS Letters 507: 25–29.
40. Bruton J, Tavi P, Aydin J, Westerblad H, Lannergren J. (2003) Mitochondrial and myoplasmic [Ca2+] in single fibres from mouse limb muscles during repeated tetanic contractions. The Journal of Physiology 551: 179–190.
41. van der Laarse WJ, Lannergren J, Diegenbach PC. (1991) Resistance to fatigue of single muscle fibres from xenopus related to succinate dehydrogenase and myofibrillar ATPase activities. Experimental Physiology 76: 589–596.
42. Celichowski J, Drzymala H. (2006) Differences between properties of male and female motor units in the rat medial gastrocnemius muscle. Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society 57: 83–93.
43. Church TS, Cheng YJ, Earnest CP, Barlow CE, Gibbons LW, et al. (2004) Exercise capacity and body composition as predictors of mortality among men with diabetes. Diabetes Care 27: 83–88.
44. Gulati M, Pandey DK, Arnsdorf MF, Lauderdale DS, Thisted RA, et al. (2003) Exercise capacity and the risk of death in women: The st james women take heart project. Circulation 108: 1554–1559.
45. Myers J, Prakash M, Froelicher V, Do D, Partington S, et al. (2002) Exercise capacity and mortality among men referred for exercise testing. The New England Journal of Medicine 346: 793–801.
46. Izquierdo M, Aguado X, Gonzalez R, Lopez JL, Hakkinen K. (1999) Maximal and explosive force production capacity and balance performance in men of different ages. European Journal of Applied Physiology and Occupational Physiology 79: 260–267.
47. Larsson L, Ansved T, Edstrom L, Gorza L, Schiaffino S. (1991) Effects of age on physiological, immunohistochemical and biochemical properties of fast-twitch single motor units in the rat. The Journal of Physiology 443: 257–275.
48. Klass M, Baudry S, Duchateau J. (2008) Age-related decline in rate of torque development is accompanied by lower maximal motor unit discharge frequency during fast contractions. Journal of Applied Physiology (Bethesda, Md.: 1985) 104: 739–746.

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PHYSICAL ACTIVITY IN ADULTHOOD: GENES AND MORTALITY

by

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Physical activity in adulthood: genes and mortality

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Observational studies report a strong inverse relationship between leisure-time physical activity and all-cause mortality. Despite suggestive evidence from population-based associations, scientists have not been able to show a beneficial effect of physical activity on the risk of death in controlled intervention studies among individuals who have been healthy at baseline. On the other hand, high cardiorespiratory fitness is known to be a strong predictor of reduced mortality, even more robust than physical activity level itself. Here, in both animals and/or human twins, we show that the same genetic factors influence physical activity levels, cardiorespiratory fitness, and risk of death. Previous observational follow-up studies in humans suggest that increasing fitness through physical activity levels could prolong life; however, our controlled interventional study with laboratory rats bred for low and high intrinsic fitness contrast with these findings. Also, we find no evidence for the suggested association using pairwise analysis among monozygotic twin pairs who are discordant in their physical activity levels. Based on both our animal and human findings, we propose that genetic pleiotropy might partly explain the frequently observed associations between high baseline physical activity and later reduced mortality in humans.

Intervention studies in humans show several positive effects of exercise on physical performance and health-related metabolism[1]. Also, in humans, observational follow-up studies report an association between high baseline levels of leisure-time physical activity and low all-cause mortality[2]. However, a causal relationship between adulthood physical activity and mortality/lifespan has not been confirmed, either in randomized controlled intervention studies, with initially healthy individuals, or in animal experiments[3–6]. High cardiorespiratory fitness is shown to be a strong predictor of reduced mortality, both in humans[7,8] and in rats[9]. Keeping in mind that fitness measurements may have higher accuracy than measurements of physical activity levels, cardiorespiratory fitness (maximal oxygen consumption, VO_2max) predicts mortality to a greater extent than physical activity level (metabolic equivalents, MET), when both are analysed in the same study[10]. Additionally, there is no consistent causal relation between (non-voluntary) occupation-related physical activity and subsequent mortality[11,12]. Thus, we lack definitive evidence on the acquired effect of physical activity on longevity. Based on past studies on elite athletes and twins, we previously suggested that genetic pleiotropy may explain at least some of the observed association between high physical activity and later morbidity or mortality[13,14]. Here, to increase our understanding of associations between genes, physical activity, and mortality, we carried out two studies. First, we conducted a controlled intervention study on the effects of voluntary running during adulthood on lifespan in two rat strains selectively bred for low and high intrinsic capacities for running (LCR and HCR, respectively). These two widely-segregated genetically heterogeneous rat strains have a 28–45% divide in median lifespan strongly predicted by a differential in VO_2max across lifespan and are thus, well-suited for testing the longevity effects accrued from voluntary physical activity[15]. We chose to study female rats, because in contrast to males, female rats are known to compensate for increased

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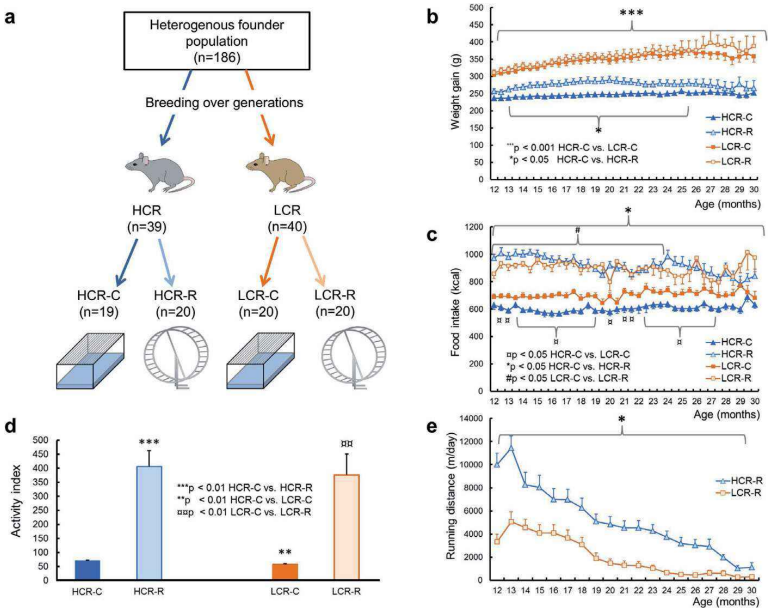


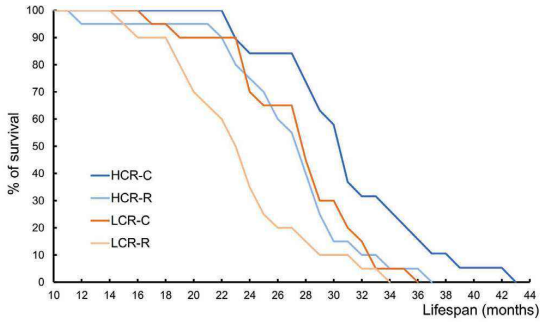
Figure 1. Rat study protocol and measurements. (**a**) Schematic of study protocol. Rats were bred for high (HCR, blue) or low (LCR, orange) intrinsic fitness, then assigned to control (C) or running (R) subgroups. (**b**) Body weights from ages 12 to 30 months. Rats per group: 12 months: HCR-C = 18, HCR-R = 15, LCR-C = 20, and LCR-R = 20; 21 months: HCR-C = 19, HCR-R = 17, LCR-C = 18, and LCR-R = 13; 30 months: HCR-C = 12, HCR-R = 8, LCR-C = 7, and LCR-R = 3. (**c**) Food intakes from ages 12 to 30 months. Rats per group: same as in **b**), except at 12 months: HCR-C = 16. (**d**) Average daily spontaneous activity measured over 3 days between 13 and 15 months of age (activity index). Rats per group: 13 months: HCR-C = 18, HCR-R = 12, LCR-C = 20, and LCR-R = 11; 15 months: HCR-C = 19, HCR-R = 13, LCR-C = 19, and LCR-R = 10. (**e**) Average running distance per day. Rats per group: 12 months: HCR-R = 15 and LCR-R = 15; 21 months: HCR-R = 16 and LCR-R = 14; 30 months: HCR-R = 4 and LCR-R = 2. Error bars represent SEMs. Figure was drawn by Sira Karvinen.

physical activity with an increase in food intake[16,17]. Thus, female rats provided the opportunity to test the independent effects of exercise on mortality, without interference from calorie restriction, which is known to increase lifespan[18].

Second, we conducted a long-term observational study in humans, with pairs of same-sex dizygotic (DZ) and monozygotic (MZ) twins. This study included a 15-y segment, where we recorded participation in physical activity, and a 23-year follow-up to evaluate mortality. Because MZ twins are genetically identical at the DNA sequence level, genetic factors were controlled in our activity-discordant, co-twin control analysis of MZ pairs. DZ twins share, on average, half of their segregating genes. Thus, genetic factors may influence physical activity discordances in DZ twin pairs. However, in most cases, both individuals in a pair of twins (MZ and DZ) share the same childhood environment; therefore, the childhood home environment is considered a controlled factor.

Results

Animal study. The HCR/LCR contrasting rat model system for intrinsic fitness capacity is the result of an over 15 year two-way artificial selection experiment described previously[19]. For the current study, we obtained 79 female rats (39 HCR and 40 LCR) and began testing when the rats were ~9 months old. Figure 1a shows the study design. We randomly assigned LCR and HCR rats to control (C) and running (R) subgroups matched within strain for body weight and fitness capacity: 19 HCR-C and 20 LCR-C, maintained in standard cages; and voluntary runners: 20 HCR-R and 20 LCR-R, maintained in cages equipped with a running wheel to permit voluntary running throughout adulthood. Figure 1b,c show details on measured body weight and food intake for all four study groups across 12–30 months of age. As previously shown, LCR rats weigh significantly more than HCR rats (age 3 months, $P < 0.001$, data not shown), and this difference remained significant throughout adult life (Fig. 1b, HCR-C vs. LCR-C, 12–30 months, $P < 0.001$). Food intake was significantly greater in runner groups compared to control for both LCR and HCR (Fig. 1c, HCR-C vs. HCR-R, 12–30 months, $P < 0.05$).



Lifespan	HCR-C	HCR-R	LCR-C	LCR-R	p
Mean	31.5 ± 5.3	26.4 ± 6.9	28.4 ± 5.5	23.8 ± 5.2	HCR-C vs. LCR-C < 0.05 HCR-C vs. HCR-R < 0.05 LCR-C vs. LCR-R < 0.01
Median	31.3	27.9	28.6	23.4	

Figure 2. Effects of genetic background and environment on lifespan. Control rats (C) had longer lifespans than rats in the runner groups (R) of the same strain (HCR-C vs. HCR-R, $P < 0.05$ and LCR-C vs. LCR-R, $P < 0.01$). Mean lifespans were also significantly different between rat strains (HCR-C vs. LCR-C, $P < 0.05$). Values in the table show means±SDs.

We measured the natural total spontaneous physical activity (horizontal and vertical movements) every three months throughout each rat's lifespan using a custom designed force-plate system[20]. The activity index was clearly higher among the runners than among the corresponding controls (Fig. 1d). Consistent with previous reports, the HCR-C group exhibited higher spontaneous activity than the LCR-C group by 23% (Fig. 1d, $P < 0.01$). In addition, for cages equipped with running wheels, the average wheel distance per day was longer in HCR-R than in the LCR-R group at time points from 12 to 29 months (Fig. 1e, $P < 0.05$).

Because physical activity is known to have positive effects on glucose metabolism, we measured insulin sensitivity at baseline (age 9 months) and after 1 year of intervention (age 21 months). At baseline, all four rat groups had similar insulin sensitivity, based on the HOMA-IR index[21]. After one year of voluntary running, the LCR-R group had a significantly lower HOMA-IR index than the LCR-C group (3.5 ± 3.0 vs. 5.7 ± 2.8, $P < 0.05$). In HCR groups, there was no difference in the HOMA index between groups (4.3 ± 3.1 vs. 4.6 ± 3.0, respectively), which is consistent with the fact that HCRs represent a healthier phenotype compared to LCRs, having lower risk of metabolic diseases even without physical training.

As expected, the Cox proportional hazards model showed that LCR rats had a higher risk of death than HCR rats, the hazard ratio (HR) for LCR compared to HCR rats adjusted for running group and age at randomization being 1.7 (95% CI: 1.1–2.6, $P = 0.028$). Interestingly, when combined, the pool of LCR and HCR runner rats had an increased risk of death compared to control groups, with a HR of 2.1 (95% CI: 1.3–3.4; $P < 0.001$). This finding persisted after adjustments for strain, age at randomization, and body weight at 9 months of age (multivariate-adjusted HR = 2.3, 95% CI: 1.4–3.6; $P < 0.001$). The decreased survivability for runners vs. controls was similar for both HCR and LCR strains. The survival curves in Fig. 2 shows the mean lifespan among runners was consistently 16% shorter than among controls in both the HCR (mean 26.4 vs. 31.5 months, $P < 0.05$) and LCR (23.8 vs. 28.4 months, $P < 0.01$) groups. The deaths caused by development of spontaneous tumours with aging did not explain the group differences in lifespan (21–45% of deaths within group).

Human study. The prospective Finnish Twin Cohort[22] includes all same-sex twin pairs born in Finland before 1958. Physical activity was measured with a structured questionnaire. We used persistence and changes in vigorous physical activity during the years 1975, 1981, and 1990 as baseline predictors of mortality. Altogether, 11 325 twin individuals (4190 complete twin pairs) answered the required physical activity questions for all three baseline time points (for more details of the cohort, see Table S1). A Cox proportional hazards model was used to analyse mortality, starting from the 1990 response date and ending July 31, 2013. Altogether, 458 deaths were observed among individuals that had performed no vigorous activity at baseline (in 1975, 1981, and 1990) and 201 deaths among individuals that persistently performed vigorous activity. An individual-based analysis showed that, compared to individuals with no vigorous activity, individuals with persistent vigorous physical activity showed decreased mortality (in 1975, 1981, and 1990); the age- and sex-adjusted HR of death was 0.55 (95% CI: 0.46–0.64) and the HR adjusted for sex, age, education level, smoking status in 1990, alcohol use (grams per day) in 1990, BMI in 1990, work activity, and health status in 1990 was 0.73 (0.61–0.88) (Fig. 3a and Table S2).

Of the 4190 same-sex twin pairs (MZ = 1388, DZ = 2547, 255 unknown zygosity), we identified 179 (4.3%) persistently discordant for participation in vigorous physical activity. These activity-discordant twin pairs comprised

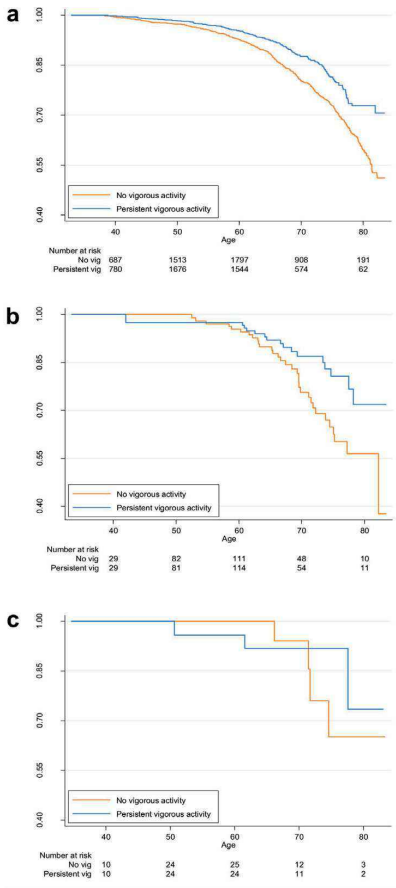


Figure 3. Kaplan-Meier survival curves of mortality in the human study. Follow-up started from the date of the 1990 questionnaire response to the end of July 2013. Groups comprised individuals with no vigorous activity (orange) vs. those with persistent vigorous activity (blue) at baseline (start of follow-up). (**a**) Survival of 2428 individuals with no vigorous activity and 2145 individuals with persistent vigorous activity; (**b**) survival of 134 discordant DZ twin pairs; (**c**) survival of 34 discordant MZ twin pairs.

2.4% (34 of 1388) of all MZ pairs and 5.3% (134 of 2547) of all DZ pairs ($P < 0.001$; Fisher's exact test for a difference in persistent discordances between MZ and DZ pairs). A pairwise analysis of these 179 twin pairs showed that the mortality HR for persistent vs. non-persistent vigorous activity was 0.65 (0.46–0.91), and after adjusting for all covariates including health status, the HR was 0.72 (0.48–1.07). Consistent with our previous twin analysis, which was based on a shorter period of physical activity discordances[23], the activity-discordant DZ pairs showed a difference in mortality (Fig. 3b, HR = 0.58, 95% CI: 0.39–0.88). However, no difference was observed in the pairwise analysis of the smaller group of activity-discordant MZ pairs (Fig. 3c, HR = 1.00, 95% CI: 0.52–1.94). The heritability of physical activity (see below) contributed to the statistical power of the analysis among MZ pairs.

To describe the total volume of physical activity performed during leisure time, we calculated a metabolic equivalent (MET) index expressed as the sum of leisure MET-hours per day at each time-point (1975, 1981, and 1990). The means of the three MET index values showed that MZ pairs (0.54) had a higher Intraclass Correlation Coefficients (ICC) than DZ pairs (0.26). This result indicates that the differences in the genetic component of the total variance influenced the long-term levels of physical activity compared to the environmental component. Using

standard techniques for genetic modelling[24] (AE model), we estimate the narrow-sense heritability for physical activity to be 53% (95% CI: 46–59%).

Due to the low number of deaths among MZ twin pairs we repeated the pairwise analyses among DZ and MZ pairs separately using cohort members who were discordant for vigorous physical activity in 1975 and 1981 (similar criteria as in our primary analysis but shorter PA discordance at baseline). This material also included older cohort members than in our primary analysis. This dataset included 778 DZ and 231 MZ vigorous activity discordant twin pairs. Among these DZ pairs there were 204 deaths and among MZ pairs 55 deaths during follow-up (between 1981 and 31st August 2013). Unadjusted pairwise HR for death among active compared to inactive members for DZ pairs was lower (HR 0.58 [95% CI 0.46–0.74]) than among MZ pairs (0.85 [0.56–1.30]. When this secondary analysis was repeated in the baseline-healthy subgroup the HRs were 0.64 (0.45–0.89) for DZ pairs and 1.05 (0.58–1.88) for MZ pairs, respectively.

We also performed an individual-based analysis of how work-related (non-voluntary) physical activity affected mortality. We found that individuals with persistently non-sedentary work had a higher risk of death than those with persistently sedentary work (age- and sex-adjusted HR = 1.15, 95% CI: 1.00–1.32; $P = 0.046$). Additional analyses that adjusted for other covariates (e.g. sex, age, education, smoking status, alcohol consumption and BMI) attenuated the HR. Also, multivariate models that analysed the persistence or change in work-related physical activity during 1975–1990 did not show any statistically significant associations with subsequent mortality. Moreover, no differences for the risk of death by work-related physical activity were observed in the pairwise analyses of all twin pairs.

Discussion

The main findings of this study along with their interpretations are summarized in Table S3. Taken together, our results are consistent with previous data on rodents[15,25,26] and humans[27,28], which indicated that genetic predisposition plays a significant role in exercise participation. These results are also consistent with our previous suggestion[14] that genetic pleiotropy may partly explain the associations observed between high physical activity and mortality in our past epidemiological studies, which called for high quality intervention studies to analyse the true effects of physical activity on morbidity and mortality among initially healthy individuals. Our results also support the notion that inherited aerobic capacity is a predictor of longevity[9,13], but further study in both animals and humans is required to determine whether this is true for the portion of aerobic capacity enhanced by vigorous physical activity. Our findings are also consistent with previous studies that show positive effects of physical activity on glucose metabolism in rodents and human twins[29,30]. However, vigorous physical activity does not improve longevity in twins[23] or rodents, particularly when commenced in maturity[3,31]. It is to note that randomized controlled trials show that vigorous physical activity has other health benefits such as improvement of both self-reported and objectively reported physical functioning and reduction of depression[1,32,33].

With this work as a foundation, we anticipate a variety of future investigations. Examples include large scale, randomized controlled intervention trials on the effects of physical activity, collaborative twin studies with larger sample sizes, Mendelian randomization analyses, and studies on genetic pleiotropy, after obtaining more knowledge on susceptibility genes. Also, there are limitations in using questionnaires as a measure of physical activity level, and future studies should combine self-reported PA data with proper objective monitoring of physical activity including specific causes of death as outcomes. In our animal experiment, divergence in physical activity started in early adulthood, which is typically the case in physical activity-discordant MZ twin pairs[30]. Our finding covers vigorous physical activity started at adulthood, but low intensity leisure-time physical activity such as walking and vigorous physical activity started during childhood may have different effects. Thus, it will be critical to determine whether physical activity affects lifespan differently when commenced early in life compared to starting later in adult life[31,34,35].

Methods

Animal study. *Animal strains.* The HCR/LCR contrasting rat model system was produced with two-way artificial selection, starting from a founder population of 186 genetically heterogeneous rats (N:NIH stock), as described previously[19] (Fig. 1a). Briefly, endurance running capacity was assessed on a treadmill and the total distance run during the test was used as a measure for intrinsic fitness capacity. Rats with the highest running capacity from each generation were bred to produce the HCR strain, and rats with the lowest capacity were bred to produce the LCR strain. For the study protocol described here, 79 female rats (39 HCR and 40 LCR) were obtained from generations 23–27. Each rat was phenotyped for fitness capacity at the University of Michigan (Ann Arbor, Michigan, USA) with a speed-ramped treadmill running test (15° slope, initial velocity of 10 m × min^{-1}, increased 1 m × min^{-1} every 2 min), when rats were ~3 months of age. Clear differences were observed in running test results (HCR 2164 ± 394 m vs. LCR 302 ± 49 m, $P < 0.001$).

After the rats arrived and habituated in Finland, we collected fasting blood samples from untrained animals (mean age 9 months), and again one year after the start of the voluntary running intervention (age 21 months). Then, each rat was carefully followed to measure lifespan. All rats were maintained in an environmentally controlled facility (12/12 h light-dark cycle, 22 °C) with water and standard food *ad libitum*.

Intervention. Before testing, up to the age of 9 months, rats were housed 2/cage in standard cages. After collecting the first blood samples (age 302 ± 38 days), each group (HCR and LCR) was divided into two subgroups matched for body weight and maximal running capacity; two subgroups were placed in standard cages (controls: HCR-C and LCR-C) and two subgroups were placed in cages with running wheels (voluntary running groups: HCR-R and LCR-R; Fig. 1a). During the voluntary running intervention, rats in all four groups were housed

1/cage. Voluntary running was followed through a computerized system throughout lifespan. Follow-up started on the day of randomization and ended at death.

Animals were euthanized when any one of the predetermined humane end point criteria were observed. These criteria were: movement disabilities, difficulty maintaining an upright position, assuming a crouched position for >48 h, labored breathing, dehydration, severe loss of body mass (>20% total body mass), chronic diarrhea or constipation for >48 h, test results that indicate loss of internal organ function, any prolonged leak from a body orifice, self-harming behaviour, and unresponsiveness to external stimuli.

Measurements of spontaneous activity. Total spontaneous physical activity was measured every three months for four days throughout each rat's lifespan. For this purpose, we used ground reaction force recordings, as described previously[20]. From that data, we calculated the activity index[20,36] as an average sum of three full days from 8 a.m. to 8 a.m. Data shown is an average value of measurements from time points 13 and 15 months of age.

Measurement of body weight and energy intake. The body weights and energy intakes of rats were followed throughout the study by weighing the rats and the amount of food consumed every second week. The energy intake was calculated based on the estimated energy content of the food, according to the manufacturer (R36, Lactamin AB).

Fasting blood samples. Fasting blood samples were collected at day time, after 5 h fasting, at ages 9 and 21 months. The group sizes were reduced between 9 and 21 months as follows: HCR-C: 19 to 17; HCR-R: 20 to 17; LCR-C: 20 to 18; and LCR-R: 20 to 15. Before collecting blood samples, all running wheels were blocked for 5 h to disable wheel movement. This action precluded any potential acute effects of running on the measured parameters. Blood glucose was measured in fresh samples (HemoCue Glucose 201 RT). Insulin was measured with ELISA from frozen (-80 °C) serum samples (Mercodia, Rat Insulin ELISA). HOMA-IR was calculated as the product of fasting glucose and insulin levels, divided by a constant[21], with the following equation:

(Glucose (mmol/l) * Insulin (μg/l))/2.430.

Ethics statement. Experimental procedures with rats conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU) and were approved by the National Animal Experiment Board, Finland (Permit number ESAVI-2010-07989/Ym-23).

Statistical Analyses. We performed statistical analyses using IBM SPSS for Windows 22.0 (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to investigate within-group normality for a given parameter of interest. Levene's test was used to assess the homogeneity of the variance assumption. When the assumptions were met, statistical comparisons of parameters between rat groups were performed with the T-test. *P*-values less than 0.05 were considered statistically significant.

Mortality analyses were conducted in individual study groups (e.g., HCR-R vs. LCR-R); in pooled groups (HCR vs. LCR); and according to training (e.g., HCR-R vs. HCR-C). Hazard ratios (HR) and 95% confidence intervals (CI) were calculated with the Cox proportional hazard model with the StataIC13 statistical package. Follow-up started on the day of randomization and ended at death. Results were adjusted for age at randomization and body weight.

Human study

Twin cohort. The Finnish Twin Cohort retains records of all same-sex twin pairs born in Finland before 1958, if both co-twins were alive in 1967[22]. In 1975, a baseline questionnaire was sent to twin pairs that were both alive at that time. A second questionnaire was sent in 1981 to all twin pairs; and a third questionnaire was sent in 1990 to all twins aged 33–60 years that had responded to at least to one of the earlier questionnaires. Among those with known addresses (93.5% of subjects) in 1975, the response rate for twin pairs was 87.6%. The response rate among respondents in 1975 that were alive in 1981 was 90.7%. The individual response rate was 77.3% in 1990[37]. The determination of zygosity was based on an accurate, validated questionnaire[38].

For the present study, inclusion criteria were: complete data on leisure-time physical activity required for calculating the metabolic equivalent (MET) index, from three postal surveys conducted in 1975, 1981, and 1990. A total of 11 325 individuals (5113 males and 6212 females) met these criteria for all three time points, including 4190 complete twin pairs (1388 MZ pairs, 2547 DZ pairs, and 255 pairs with unknown zygosity). The physical activity levels and mortality of twins did not differ between pairs with one respondent (ineligible for pairwise analyses) and pairs with two respondents (eligible for pairwise analyses).

Assessment of predictors and potentially confounding variables. The questionnaires mailed in 1975, 1981, and 1990 included questions on physical activity performed during leisure time, work-related physical activity, weight, height, occupation, alcohol use, smoking, and physician-diagnosed diseases. For the current study, participation in vigorous leisure time physical activity (voluntary) and work-related physical activity (less voluntary) in 1975, 1981, and 1990 were used as separate baseline predictors of mortality.

Leisure-time physical activity. Leisure physical activity habits were assessed with identical validated questions on surveys conducted in 1975 and 1981[39,40] and with modified questions on the survey conducted in 1990[41]. Assessments of vigorous physical activity were based on a single set of intensity categories applied at all three time points; these were: 1) walking, 2) alternately walking and jogging, 3) jogging (light running), and 4) running. Vigorous activity was defined as levels 2, 3, or 4 (all more intensive than normal walking), performed at least 3–5 times/month in 1975 and 1981, or at least for 30 min each week in 1990. Then, a five-category vigorous activity

variable was created to assess changes/persistence over time. Category 1 was 'persistent vigorous activity', defined as vigorously active at all three time points (1975, 1981, and 1990); conversely, category 5 was 'no vigorous activity' defined as no vigorous activity at all three time points. Intermediate categories were mixed participation in vigorous activity over time (1975, 1981, 1990, respectively), as follows: 'increased' (no, no/yes, yes), 'decreased' (yes, yes/no, no), and 'changed' ([yes, no, yes] or [no, yes, no]). This five-category vigorous activity variable was used as the main mortality predictor for the human study.

To characterize the amount of long-term leisure time physical activity performed, we calculated the MET index, based on a series of structured questions that covered leisure physical activity and any physical activity performed during the journey to and from work[39,41]. We calculated the activity MET index by assigning a multiple of resting metabolic rate to each activity and by calculating intensity x duration x frequency of activity. The MET index was expressed as the sum of leisure MET-hours per day at each time-point (1975, 1981, and 1990). Finally, we calculated a mean MET index for long-term leisure-time physical activity; i.e., the MET indexes for 1975, 1981, and 1990 were added together and divided by 3.

Work-related physical activity. Work-related physical activity was a categorical variable evaluated with a four-point ordinal scale[23]. The question was as follows: "What kind of work did/do you do in your present job or the job you last had?" The answers were: 1) mainly sedentary work, which requires very little physical activity; 2) work that involves standing and walking, but no other physical activity; 3) work that requires standing and walking, and in addition, requires lifting and carrying; or 4) heavy physical work. Answer 1 was classified as sedentary work, and answers 2, 3, or 4 were classified as non-sedentary work. Then, a five-category work-related activity variable was created that covered the three measurement points, as described for participation in vigorous leisure-time physical activity (see above). Those categories were: persistent sedentary work, persistent non-sedentary work, increased, decreased, or changed work-related physical activity.

Confounding variables. For the present study, body mass index (BMI), smoking status, use of alcohol, health status (all reported in 1990), and self-reported education level (reported in 1981) were tested as covariates. The level of education was assessed in the 1981 questionnaire by asking what kind of schooling the respondent had completed[37]. The answers were converted into a nine-class variable of years of schooling for the analyses. BMI (kg/m^2) was calculated from self-reported height and weight. Smoking status was determined from responses to detailed questions on smoking history, coded into four categories (never smoked, former smoker, occasional smoker, and current (daily) smoker)[42]. Alcohol use was expressed in units of alcohol grams consumed per day[43]. According to the 1990 questionnaire, individuals were considered to have a somatic illness, when he/she had (i) any self-reported disease diagnosed by a physician, or (ii) a self-reported life event related to a serious injury or illness, or (iii) a self-reported permanent work disability[37]. Other subjects were classified as healthy. The cohort included a total of 4846 twins who were healthy in 1990; 91.4% of them had lived together with their co-twin until the age of 16 years.

Ethics statement. An earlier statement from the ethics committee of the Department of Public Health, University of Helsinki covered our current study. The study was conducted according to the declaration of Helsinki. All the participants gave their informed consent by providing questionnaire responses. The participants were provided regular feedback on the purpose and conduct of the study and were informed that they may withdraw from the study at any time.

Statistical analysis. Mplus version 7 was used to estimate Intraclass Correlation Coefficients (ICC) and to partition the total variance into genetic and environmental components[44] with standard techniques[24]. All-cause mortality during follow-up was analysed. The exact dates of death, causes of death, and emigration from Finland were available from the Population Register Centre of Finland. A total of 243 177 person-years were accumulated during follow-up, from the 1990 individual response date to the end of July 2013, the date of emigration, or death. During that period, 1478 individuals died at a mean age of 63.6 years (range 37.1 to 82.8 years). First, we analysed individual-based mortality in relation to leisure-time physical activity participation, adjusted for age and sex. We calculated HRs and 95% CIs with the Cox proportional hazard model, and we applied clustering for family members. Next, we adjusted the model for baseline levels of education, smoking status, alcohol use, BMI, and work-related activity, by adding one covariate at a time to the model. These adjustments did not significantly change the HRs; therefore, three different models are shown in the Table S2. Additionally, a fourth model is shown, where health status was added to the full model. Individuals that did not participate in vigorous activity were used as the reference group in all analyses of leisure time physical activity. In addition, pairwise analyses were performed with the same models for all twin pairs. We analysed all twins at once, and then, MZ and DZ pairs separately. We used the identical analysis strategy for assessing work-related physical activity categories, except we did not adjust for work-related physical activity. Data were analysed with the StataIC13 statistical package (Stata Corp, College Station, Texas, U.S.A.).

References

1. Physical Activity Guidelines Advisory Committee. Physical Activity Guidelines Advisory Committee Report. Washington, DC: U.S. Department of Health and Human Services (2008).
2. Löllgen, H., Bockenhoff, A. & Knapp, G. Physical activity and all-cause mortality: an updated meta-analysis with different intensity categories. *Int. J. Sports Med.* **30,** 213–224 (2009).
3. Goodrick, C. L., Ingram, D. K., Reynolds, M. A., Freeman, J. R. & Cider, N. L. Differential effects of intermittent feeding and voluntary exercise on body weight and lifespan in adult rats. *J. Gerontol.* **38,** 36–45 (1983).
4. Samorajski, T. *et al.* Effect of exercise on longevity, body weight, locomotor performance, and passive-avoidance memory of C57BL/6J mice. *Neurobiol. Aging* **6,** 17–24 (1985).

5. Vaanholt, L. M., Daan, S., Garland, T. Jr & Visser, G. H. Exercising for life? Energy metabolism, body composition, and longevity in mice exercising at different intensities. *Physiol. Biochem. Zool.* **83,** 239–251 (2010).
6. Garcia-Valles, R. *et al.* Life-long spontaneous exercise does not prolong lifespan but improves health span in mice. *Longev Healthspan* **2,** 14-2395-2-14 (2013).
7. Myers, J. *et al.* Exercise capacity and mortality among men referred for exercise testing. *N. Engl. J. Med.* **346,** 793–801 (2002).
8. Kodama, S. *et al.* Cardiorespiratory fitness as a quantitative predictor of all-cause mortality and cardiovascular events in healthy men and women: a meta-analysis. *JAMA* **301,** 2024–2035 (2009).
9. Koch, L. G. *et al.* Intrinsic Aerobic Capacity Sets a Divide for Aging and Longevity. *Circ. Res.* **109,** 1162–1172 (2011).
10. Lee, D. C. *et al.* Comparisons of leisure-time physical activity and cardiorespiratory fitness as predictors of all-cause mortality in men and women. *Br. J. Sports Med.* **45,** 504–510 (2011).
11. Holtermann, A., Marott, J. L., Gyntelberg, F., Sogaard, K., Suadicani, P., Mortensen, O. S. *et al.* Occupational and leisure time physical activity: risk of all-cause mortality and myocardial infarction in the Copenhagen City Heart Study. A prospective cohort study. BMJ Open. 2012 Feb 13;2(1):e000556,2011-000556. Print 2012.
12. Richard, A., Martin, B., Wanner, M., Eichholzer, M. & Rohrmann, S. Effects of leisure-time and occupational physical activity on total mortality risk in NHANES III according to sex, ethnicity, central obesity, and age. *J. Phys. Act. Health*. **12,** 184–192 (2015).
13. Kujala, U. M. *et al.* Occurrence of chronic disease in former top-level athletes. Predominance of benefits, risks or selection effects? *Sports Med*. **33,** 553–561 (2003).
14. Kujala, U. M. Physical activity, genes, and lifetime predisposition to chronic diseases. *Eur. Rev. Aging Phys. Act*. **8,** 31–36 (2011).
15. Koch, L. G., Britton, S. L. & Wisloff, U. A rat model system to study complex disease risks, fitness, aging, and longevity. *Trends Cardiovasc. Med*. **22,** 29–34 (2012).
16. Titchenal, C. A. Exercise and food intake. What is the relationship? *Sports Med*. **6,** 135–145 (1988).
17. Holloszy, J. O. Exercise Increases Average Longevity of Female Rats Despite Increased Food Intake and No Growth Retardation. *Journal of Gerontology* **48,** B97–B100 (1993).
18. Goodrick, C. L., Ingram, D. K., Reynolds, M. A., Freeman, J. R. & Cider, N. L. Effects of intermittent feeding upon growth and life span in rats. *Gerontology* **28,** 233–241 (1982).
19. Koch, L. G. & Britton, S. L. Artificial selection for intrinsic aerobic endurance running capacity in rats. *Physiol. Genomics* **5,** 45–52 (2001).
20. Silvennoinen, M., Rantalainen, T. & Kainulainen, H. Validation of a method to measure total spontaneous physical activity of sedentary and voluntary running mice. *J. Neurosci. Methods* **235,** 51–58 (2014).
21. Cacho, J., Sevillano, J., de Castro, J., Herrera, E. & Ramos, M. P. Validation of simple indexes to assess insulin sensitivity during pregnancy in Wistar and Sprague-Dawley rats. *Am. J. Physiol. Endocrinol. Metab*. **295,** E1269–76 (2008).
22. Kaprio, J. & Koskenvuo, M. Genetic and environmental factors in complex diseases: the older Finnish Twin Cohort. *Twin Res.* **5,** 358–365 (2002).
23. Kujala, U. M., Kaprio, J. & Koskenvuo, M. Modifiable risk factors as predictors of all-cause mortality: the roles of genetics and childhood environment. *Am. J. Epidemiol.* **156,** 985–993 (2002).
24. Neale, M. C. & Cardon, L. R. *Methodology for Genetic Studies of Twins and Families*. Dordrecht: Kluwer Academic Publishers (1992).
25. Kelly, S. A. *et al.* Genetic architecture of voluntary exercise in an advanced intercross line of mice. *Physiol. Genomics* **42,** 190–200 (2010).
26. Novak, C. M. *et al.* Spontaneous activity, economy of activity, and resistance to diet-induced obesity in rats bred for high intrinsic aerobic capacity. *Horm. Behav.* **58,** 355–367 (2010).
27. Stubbe, J. H. *et al.* Genetic influences on exercise participation in 37,051 twin pairs from seven countries. *PLoS One* **1,** e22 (2006).
28. de Geus, E. J., Bartels, M., Kaprio, J., Lightfoot, J. T. & Thomis, M. Genetics of regular exercise and sedentary behaviors. *Twin Res. Hum. Genet*. **17,** 262–271 (2014).
29. Changsun, K *et al.* The effects of aerobic treadmill exercise training on insulin resistance and bone metabolic turnover in diabetes mellitus rats. *J Exerc Nutr Biochem* **17,** 61–69 (2013).
30. Rottensteiner, M. *et al.* Physical activity, fitness, glucose homeostasis, and brain morphology in twins. *Med. Sci. Sports Exerc.* **47,** 509–518 (2015).
31. Edington, D. W., Cosmas, A. C. & McCafferty, W. B. Exercise and longevity: Evidence for a threshold age. *Jornal of Gerontology* **27,** 341 (1972).
32. Kujala UM. Evidence of the effects of exercise therapy in the treatment of chronic disease. *Br J Sports Med* **43,** 550–5 (2009).
33. Herring, M. P., Puetz, T. W., O'Connor, P. J. & Dishman, R. K. Effect of exercise training on depressive symptoms among patients with a chronic illness: a systematic review and meta-analysis of randomized controlled trials. *Arch. Intern. Med*. **172,** 101–111 (2012).
34. Goodrick, C. L. Effects of long-term voluntary wheel exercise on male and female Wistar rats. I. Longevity, body weight, and metabolic rate. *Gerontology* **26,** 22–33 (1980).
35. Holloszy, J. O., Smith, E. K., Vining, M. & Adams, S. Effect of voluntary exercise on longevity of rats. *J. Appl. Physiol. (1985)* **59,** 826–831 (1985).
36. Biesiadecki, B. J., Brand, P. H., Koch, L. G. & Britton, S. L. A gravimetric method for the measurement of total spontaneous activity in rats. *Proc. Soc. Exp. Biol. Med.* **222,** 65–69 (1999).
37. Romanov, K., Varjonen, J., Kaprio, J. & Koskenvuo, M. Life events and depressiveness - the effect of adjustment for psychosocial factors, somatic health and genetic liability. *Acta Psychiatr. Scand.* **107,** 25–33 (2003).
38. Sarna, S., Kaprio, J., Sistonen, P. & Koskenvuo, M. Diagnosis of twin zygosity by mailed questionnaire. *Hum. Hered.* **28,** 241–254 (1978).
39. Kujala, U. M., Kaprio, J., Sarna, S. & Koskenvuo, M. Relationship of leisure-time physical activity and mortality: The finnish twin cohort. *JAMA* **279,** 440–444 (1998).
40. Waller, K., Kaprio, J. & Kujala, U. M. Associations between long-term physical activity, waist circumference and weight gain: a 30-year longitudinal twin study. *Int. J. Obes. (Lond)* **32,** 353–361 (2008).
41. Lahti, J., Laaksonen, M., Lahelma, E. & Rahkonen, O. The impact of physical activity on physical health functioning-a prospective study among middle-aged employees. *Prev. Med.* **50,** 246–250 (2010).
42. Kaprio, J. & Koskenvuo, M. A prospective study of psychological and socioeconomic characteristics, health behavior and morbidity in cigarette smokers prior to quitting compared to persistent smokers and non-smokers. *J. Clin. Epidemiol.* **41,** 139–150 (1988).
43. Kaprio, J. *et al.* Genetic influences on use and abuse of alcohol: a study of 5638 adult Finnish twin brothers. *Alcohol. Clin. Exp. Res.* **11,** 349–356 (1987).
44. Muthén, L. K. & Muthén, B. O. *Mplus User's Guide*. Sixth Edition. Los Angeles, CA: Muthén & Muthén (1998-2012).

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Author Contributions

H.K. and U.M.K. designed the study. S.K., K.W., H.K. and U.M.K. drafted the manuscript. S.K., K.W. and U.M.K. analysed the data. H.K. led the animal experiment. J.K. led the twin cohort data collection. S.K. performed the animal experiment. M.S. participated in planning the animal experiment, set up the voluntary running and spontaneous activity measurement systems, and assisted in collecting the blood samples. S.L.B. and L.G.K. bred and phenotyped the animals. All authors contributed to the revision of the manuscript and approved the final version of the manuscript.

Additional Information

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III

EFFECTS OF INTRINSIC AEROBIC CAPACITY, AGING AND VOLUNTARY RUNNING ON SKELETAL MUSCLE SIRTUINS AND HEAT SHOCK PROTEINS

by

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Effects of intrinsic aerobic capacity, aging and voluntary running on skeletal muscle sirtuins and heat shock proteins

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ABSTRACT

Aim: Sirtuins are proteins that connect energy metabolism, oxidative stress and aging. Expression of heat shock proteins (Hsps) is regulated by heat shock factors (HSFs) in response to various environmental and physiological stresses, such as oxidative stress. Oxidative stress accumulates during aging which makes cells more prone to DNA damage. Although many experimental animal models have been designed to study the effects of knockdown or overexpression of sirtuins, HSFs and Hsps, little is known about how aging *per se* affects their expression. Here we study the impact of intrinsic aerobic capacity, aging and voluntary exercise on the levels of sirtuins, HSFs and Hsps in skeletal muscle.
Methods: We studied the protein levels of sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7), HSF1, HSF2, Hsp10, Hsp27 and Hsp70 before and after one-year of voluntary running intervention of rat strains selectively bred for intrinsic aerobic exercise capacity; high capacity runners (HCR) and low capacity runners (LCR) differ by more than 30% for median lifespan. This setup enabled us to discern the effects of inborn aerobic capacity, aging and exercise activity on the protein levels of sirtuins, HSFs and Hsps in skeletal muscle.
Results: Our results revealed that the longer lived HCR rats had higher SIRT3, HSF1 and HSF2 contents in skeletal muscle (*gastrocnemius*, $p < 0.05$) than LCRs. Neither aging nor voluntary running had a significant effect on the studied sirtuin proteins. Aging significantly increased the protein levels of HSF1, HSF2 and Hsp27 ($p < 0.05$).
Conclusion: Our finding of elevated SIRT3 levels in HCR rats is in line with previous studies; SIRT3 in general is linked to elevated fatty acid oxidation and oxidative phosphorylation, which previously have been associated with metabolic profile of HCRs. HSF1, HSF2 and Hsp27 levels increased with aging, showing that aged muscles responded to aging-related stress. Our study shows for the first time that SIRT3 protein level is linked to high inborn aerobic capacity, and may be directly interconnected to longevity.

1. Introduction

Aging is a physiological phenomenon during which a progressive decline of organ and tissue function is accompanied with the development of complex diseases. Since aging phenotypes at whole body level results from a complex set of changes at the cellular level, several theories have been formulated to address the underlying mechanisms behind this phenomenon. Telomere shortening and accumulation of oxidative stress are among the most prominent candidates of these theories (Wei and Lee, 2002; Van Remmen and Richardson, 2001; Kruk et al., 1995; Olovnikov, 1996). In yeast cells, the SIRT2 ortholog Sir2 was shown to promote longevity, which raised the interest towards a possible role of sirtuin proteins in mammalian aging (Kaeberlein et al., 1999).

Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent protein deacylases that link protein acylation, metabolism and aging (Guarente, 2013). Mammals have seven sirtuins; SIRT3, SIRT4 and SIRT5 are mitochondrial, SIRT1, SIRT6 and SIRT7 are primarily nuclear, and SIRT2 is found both in the nucleus and the cytoplasm (Finkel et al., 2009; Verdin et al., 2010). Generally, the sirtuins deacylate proteins that are involved either in i) response to oxidative stress or ii) control of metabolism (Guarente, 2013). Sirtuins interact with telomeric chromatin and several components of the DNA repair machinery making them potential targets for studies on the mechanisms of oxidative stress in aging (Nakagawa and Guarente, 2011; Michishita et al., 2008). Sirtuins are also energy sensor proteins that respond

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strongly to caloric restriction, a well-recognized intervention to increase life span of lower model organisms and rodents as well as primates (Bishop and Guarente, 2007; Rogina and Helfand, 2004; Bodkin et al., 2003). Furthermore, sirtuins play a role in the selection of energy source by regulating fatty acid oxidation (Hirschey et al., 2010; Nasrin et al., 2010) along with mitochondrial energy metabolism and ATP homeostasis (Ho et al., 2013; Park et al., 2013).

Downstream from sirtuins, heat shock factors (HSFs) are important transcription factors for protecting cells from protein-damaging stress associated with misfolded proteins, and therefore aging and aging related diseases (Rodgers et al., 2005; Westerheide et al., 2009). HSFs activate the transcription of a large number of genes that regulate protein homeostasis, including genes encoding heat shock proteins Hsp27 and Hsp70 (Westerheide et al., 2009; Vihervaara et al., 2013). Hsp10 typically functions as chaperone in mitochondria together with Hsp60 (Jia et al., 2011). In general, Hsp proteins function as molecular chaperones by supporting the folding of newly synthesized polypeptides and the assembly of multi-protein complexes (Beckmann et al., 1990; Kampinga et al., 2009; Jakob et al., 1993). SIRT1 has been shown to maintain the DNA-binding state of HSF1 for Hsp70 induction (Liu et al., 2014) whereas Hsp10 has proven to be a functional SIRT3 substrate (Lu et al., 2015).

Several experimental animal models have been genetically designed to study the effects of knockdown of sirtuin and HSF proteins. SIRT6-knockout mice have aging-like phenotype, including severe hypoglycemia and loss of subcutaneous fat, leading to death at early age (Mostoslavsky et al., 2006; Xiao et al., 2010). SIRT7-knockout mice display cardiac hypertrophy (Vakhrusheva et al., 2008), whereas SIRT2-knockdown mice develop tumorigenesis, leading to the notion that SIRT2 acts as a tumor suppressor (Park et al., 2012). Mice lacking both SIRT3 alleles have higher levels of fatty-acid oxidation intermediate products and triglycerides during fasting, associated with decreased levels of fatty-acid oxidation, compared to livers from wild-type mice (Hirschey et al., 2010). Interestingly, reduced SIRT4 levels significantly increase fatty acid oxidation and cellular respiration in myotubes, demonstrating that that SIRT4 inhibition increases fat oxidative capacity and mitochondrial function in muscle (Nasrin et al., 2010). SIRT5-knockout mice are hyperammonemic during fasting and SIRT5-overexpressing mice have conversely increased urea production (Nakagawa et al., 2009). SIRT1-knockout mice are not viable in inbred strain backgrounds and show pleiotropic phenotypes in outcrossed lines, including small size, developmental defects and sterility (McBurney et al., 2003). In mice overexpression of SIRT1 leads to similar phenotype as calorie-restricted diet: mice are leaner, more metabolically active and display reductions in blood cholesterol, insulin and fasting glucose compared to littermate controls (Bordone et al., 2007). In mammals, HSFs are involved in several developmental pathways, and HSF1-knockout mice exhibit growth retardation, prenatal lethality and decreased lifespan (Xiao et al., 1999). Taken together, the knockout animal models of sirtuins and HSFs show several divergent metabolic defects of which some lead to a shortened lifespan.

Previous studies have revealed that high aerobic capacity and/or high physical activity levels are strongly associated with long lifespan in humans (Lollgen et al., 2009; Myers et al., 2002). Recently, it was also reported that high intrinsic aerobic capacity is strongly linked to longevity in rodents (Koch et al., 2011). Because of this strong statistical linkage between exercise capacity and survivability in both rats and humans, many mechanistic studies have concentrated on the effects of exercise on sirtuins and Hsps. It has been shown that prolonged exercise training increases SIRT1 activity in heart tissue in aged rats (Ferrara et al., 2008). SIRT3 has been reported to be upregulated by exercise and chronic muscle contractions (Gurd et al., 2012; Palacios et al., 2009), and resistance training has been shown to increase Hsp levels in skeletal muscle of both young and old rats (Murlasits et al., 2006). To date, SIRT1 and SIRT6 have proven to be strong determinants of life span in mice (Kanfi et al., 2012; Satoh et al., 2013). However, only a few studies have addressed how healthy aging itself affects the expression of sirtuins, HSFs and Hsps. There are indications that the activity of sirtuins declines during aging (Nakagawa and Guarente, 2011), yet more animal and humans studies are needed to explore the effects of the whole sirtuin protein family on lifespan (Jia et al., 2012).

Here we use rat strains that differ from their intrinsic aerobic capacity; high capacity runners (HCR) and low capacity runners (LCR) (Koch and Britton, 2001) before and after one-year voluntary running intervention to measure the proteins levels of sirtuins, HSFs and Hsps. This setup enabled us to investigate the effects of inborn aerobic capacity, aging *per se* and exercise on skeletal muscle sirtuin, HSF, and Hsp protein levels. This animal model is well suited for aging studies, since in addition to differing from their aerobic capacity HCRs have been shown to have 28-48% longer lifespan than LCRs (Koch et al., 2011). We hypothesized that HCRs display higher expression of sirtuin, HSF and Hsp proteins compared to LCRs due to their verified longer median lifespan that is tightly coupled to maximal aerobic capacity and their presumed better capability to compensate aging-related oxidative stress. We also hypothesized that the studied protein levels are higher in HCRs in response to exercise as HCRs have been reported to engage in more voluntary activity on a running wheel and to be spontaneously more active in novel environments than LCRs (Koch et al., 2011; Novak et al., 2010a; Waters et al., 2008).

2. Materials and methods

2.1. Rat strains

The HCR/LCR contrasting rat model system was produced via two-way artificial selection, starting from a large founder population of genetically heterogeneous rats (N:NIH stock), as described previously (Koch and Britton, 2001). The phenotype for endurance running capacity was assessed at the University of Michigan (Ann Arbor, Michigan, USA) with a speed-ramped treadmill running test (15° slope, initial velocity of 10 m min^{-1}, increased 1 m min every 2 min) when the rats were 11 weeks of age. For this study, 60 female rats (30 HCRs and 30 LCRs) produced at generations 23-27 of selection were used. All rats were housed in an environment controlled facility (12/12 h light-dark cycle, 22°C) and received water and standard rodent feed (R36, Labfor, Stockholm, Sweden) *ad libitum*.

2.2. Protocol

We conducted similar speed-ramped maximal treadmill running tests to the rats as described above at the age of 9 and 21 months and collected skeletal muscle samples (gastrocnemius muscle) at the same time points (Fig. 1). Before the first measurements and sampling, the rats were housed in pairs in standard cages. After the first measurements rats were divided evenly into weight and maximal running capacity matched groups ($n = 10$) and randomly assigned to control (standard cage) vs voluntary running groups (cage with an access to a running wheel). We collected samples from the non-trained animals at time points that coincided with before (age 9 months) and after one-year voluntary running intervention (age 21 months). All together, we had six different subgroups of rats; HCR_before (no intervention), LCR_before (no intervention), HCR-C_after (control), HCR-R_after (runner), LCR-C_after (control) and LCR-R_after (runner) (Fig. 1). During this one-year intervention the rats were housed 1/cage. Rats in the control groups had a wooden tunnel and all rats had nesting material to enrich their environment.

2.3. Body weight and energy intake

Body weight and energy intake of the rats were followed throughout the one-year intervention by weighing the rats and consumed food

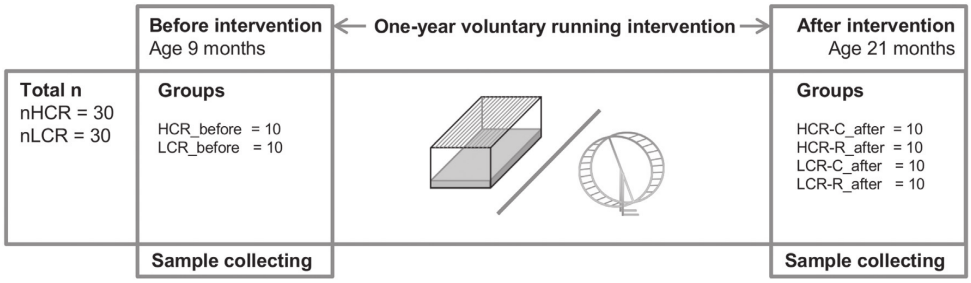

Fig. 1. Schematic representation of the study protocol. Before the voluntary running intervention we collected samples from HCR/LCR rats from untrained state (HCR_before and LCR_before, n = 10/group). We had one-year voluntary running intervention with 4 sub-groups: HCR-C (control), HCR-R (runner), LCR-C (control) and LCR-R (runner) from which we collected samples after the intervention (n = 10/group).

every second week. The energy intake was calculated from the feed energy content information provided by the manufacturer (Labfor).

2.4. Voluntary running distance and spontaneous activity

Voluntary running distance from the running wheels was followed throughout the one-year intervention with a computerized recording system. Running wheel was mounted on a cage (Techniplast 2154F0105, Buguggiate, Italy), and the wheel was connected to a computerized recording system (Acer Verinton 6900Pro, 32 bit processor produced by Intel, Windows XP). Total wheel laps were recorded continuously, and the total running distance per day was determined by multiplying the number of wheel rotations by the circumference of the running wheel (Ø 34.5 cm). From daily running distances we calculated an average daily running distance for every two-week period. Spontaneous activity was measured for four days at two time points; 13 and 15 months of age. For this purpose, we used ground reaction force recordings, as described previously (Silvennoinen et al., 2014). The absolute values of the differences between consecutive force values were calculated as described by Silvennoinen *et al* (Silvennoinen et al., 2014). The mean of the absolute values were calculated from every second from total 20 values per second. To obtain a single value for total spontaneous activity, the 1-s means were summed for the total measurement time and the sum was divided by the body mass (kg) of the measured rat. From that data, we calculated the activity index as a sum on three day activity per each month (Silvennoinen et al., 2014; Biesiadecki et al., 1999).

2.5. Tissue processing

Gastrocnemius muscle (n = 10/group) samples were collected from HCR/LCR rats before and after one-year voluntary running intervention. Distal part of gastrocnemius muscle (2/3 of total muscle volume) was used for the analyses. The snap frozen samples were homogenized in liquid nitrogen and processed with either of the following protocols:

2.5.1. Sirtuins, Hsp10, PGC1a, cyt C and citrate synthase

The homogenized muscle sample was dissolved in ice-cold buffer (20 mM HEPES [pH 7.4], 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM, b-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% NP-40 [nonyl phenoxypolyethoxylethanol], 0.2% sodium deoxycholate, and 3% protease and phosphatase inhibitor cocktail [P 78443; Pierce, Rockford, IL]). The muscle homogenate was then centrifuged at 10.000 x *g* for 10 min at 4°C. Total protein content was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) with an automated KoneLab instrument (Thermo Scientific, Vantaa, Finland).

2.5.2. HSFs, Hsp27and Hsp70

The muscle homogenates were lysed in lysis buffer (24 mM HEPES [pH 7.4], 100 mM NaCl, 5 mM EDTA, 0.5% Triton-X-100, 200 mM β-glycerolphosphate, 20 mM PNPP, 100 μM ortovanadate), and protein concentration was measured with the Bradford method.

2.6. Citrate synthase activity

Citrate synthase activity ($U \bullet \mu g^{-1} \bullet min^{-1}$) in the gastrocnemius muscle (n = 10/group) was studied as it is used as a marker of aerobic capacity and mitochondrial density in skeletal muscle (Hoppeler, 1986). Citrate synthase activity was measured from the same muscle homogenates that were used to determine the total protein content (Citrate Synthase Assay Kit Sigma-Aldrich) with an automated KoneLab instrument (Thermo Scientific).

2.7. Western blot analyses

2.7.1. Sirtuins, Hsp10, PGC1a, cyt C and OXPHOS Cocktail

Aliquots of muscle homogenate were solubilized in Laemmli sample buffer and heated at 95°C to denaturate proteins. Samples containing 30 μg of total protein were separated by SDS-PAGE for 60-90 min at 200 V using 4–20% gradient gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to PVDF membranes at 300 mA constant current for 2 h on ice at 4°C. The homogeneity of protein loading was checked by staining the membrane with Ponceau S. Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk for 2 h and then incubated overnight at 4°C with commercially available polyclonal primary antibodies to measure the following protein contents with stated dilutions: SIRT1 (1:2000; ab28170, Abcam), SIRT2 (1:500; ab75436, Abcam), SIRT3 (1:800; ab118334, Abcam), SIRT4 (1:1000; ab124521, Abcam), SIRT5 (1:1000; ab13697, Abcam) SIRT6 (1:500; ab62739, Abcam), SIRT7 (1:500; 13477, Cayman Chemical Company), Hsp10 (1:2000; SAB4501465, Sigma), PGC-1α (1:4000; 516557, Calbiochem), cytochrome C (cyt C) (1:500; sc-8385, Santa Cruz biotechnology, Inc.), Total OXPHOS Cocktail (1:1000; ab110413; Abcam) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000; ab9485, Abcam). After the primary antibody incubation membranes were washed in TBS-T, incubated with suitable secondary antibody diluted in TBS-T with 2.5% milk for 1 h followed by washing in TBS-T. Proteins were visualized by ECL according to the manufacturer's protocol (SuperSignal West femto maximum sensitivity substrate, Pierce Biotechnology) and quantified using ChemiDoc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories). Sirtuin protein and OXPHOS Cocktail levels were normalized to corresponding Ponceau S stained actin band and after this the mitochondrial sirtuins (SIRT3, SIRT4 and SIRT5) were normalized to OXPHOS Cocktail level. Mitochondrial Hsp10 was first normalized to corresponding GAPDH band and

thereafter to OXPHOS Cocktail level similarly as mitochondrial sirtuins. PGC-1α and cyt C were normalized to corresponding GAPDH band.

2.7.2. HSFs and Hsp27and Hsp70

Samples containing 35 μg of total protein were separated by SDS-PAGE on 7.5 % gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes with the semi-dry method. Transfer efficiency was checked with Ponceau S staining, and the membranes were boiled for 15 min before being blocked with 5% milk in PBS-0.3%-Tween 20 and immunoblotted with the following primary antibodies: α-HSF1 (1:1000; SPA-901, Enzo Life Sciences), α-HSF2 (1:200; Clone 3E2, Cayman Chemicals), α-HSP70 (1:5000; SPA-810, Enzo Life Sciences), α-HSP27 (1:1000; SPA-800, Enzo Life Sciences), α-GAPDH (1: 15000; ab9485, Abcam). After the primary antibody incubation membranes were washed in PBS-T and incubated with suitable secondary antibody. The signal was developed using Amersham ECL Plus substrate (GE Healthcare) and quantified using ChemiDoc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories). HSF and Hsp protein levels were normalized to corresponding GAPDH band.

2.8. Ethics statement

This study was approved by the National Animal Experiment Board, Finland (Permit number ESAVI-2010-07989/Ym-23).

2.9. Conflict of interest

The authors declare no conflict of interest.

2.10. Statistical analyses

Statistical analyses for all variables were carried out using SPSS for Windows 22 statistical software (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to investigate within group normality for a given parameter of interest. Levene's test was conducted to assess the homogeneity of the variance assumption. When the normality or equality of variance assumptions were not met, statistical comparisons of parameters between LCR and HCR groups were made using Mann-Whitney test. Univariate analysis was done by combining the data from both studied rat strains to analyze the strain (HCR or LCR), age (before [age 9 months] or after [age 21 months] the intervention) and running (access to running wheel or a standard cage) effects to the measured parameters. P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Body weight and energy intake

LCRs were heavier during the whole intervention compared to the corresponding HCRs in both studied groups (LCR-C vs. HCR-C and LCR-R vs. HCR-R, $p < 0.05$, Fig. 2A). Energy intake was greater in runner groups compared to the corresponding controls in both rat strains ($p < 0.05$, Fig. 2B). In HCRs, HCR-R consumed more feed compared to HCR-C during 11.5-20 months, whereas in LCRs, LCR-R had higher energy intake during 11.5-12 and 13.5-20 months ($p < 0.05$). The control groups of both rat strains had a similar energy intake.

3.2. Voluntary running and spontaneous activity

HCRs run more voluntarily compared to the LCR runners during the whole intervention ($p < 0.05$, Fig. 3A). HCRs also had higher spontaneous activity compared to LCRs during control and running intervention, showing a strain effect at time point 15 months ($p < 0.050$, Fig. 3B). Rats from both strains that had the opportunity to voluntary run had a clearly higher spontaneous activity at both studied time points than the corresponding controls (Running effect $p < 0.05$, Fig. 3B).

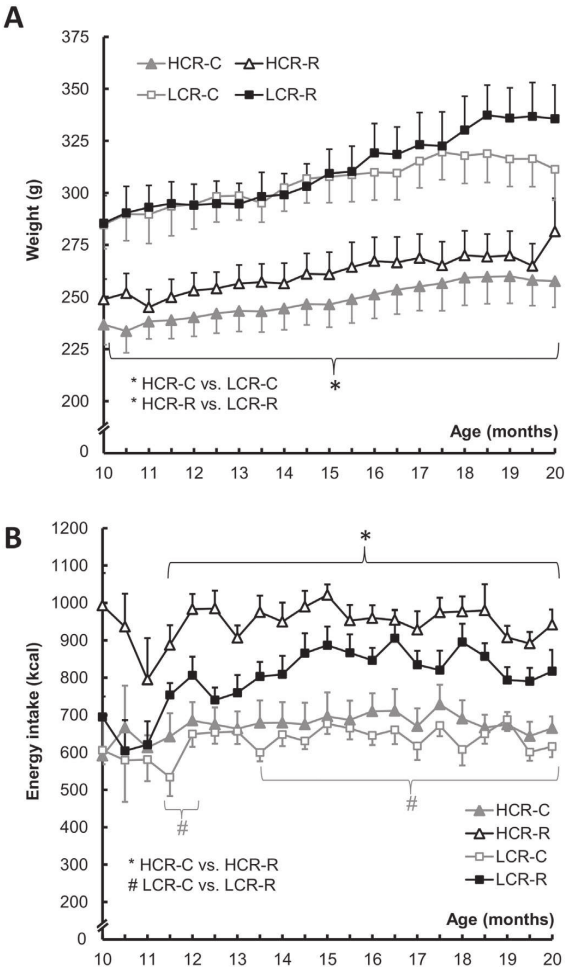

Fig. 2. Body weight and energy intake during one-year intervention. n = 10/group. *, #$p < 0.05$, Values are expressed as mean ± SEM.

3.3. Western blot analyses

Univariate analysis showed a strain effect for SIRT3 on the gastrocnemius muscle with HCRs having higher protein level compared to LCRs ($p < 0.050$, Fig. 4A, Table 1). Also, Hsp10 normalized to GAPDH showed a strain effect with HCRs having higher protein level than LCRs ($p < 0.010$, *data not shown*). Strain, aging or voluntary running had no significant effect on other studied sirtuins or Hsp10 normalized to OXPHOS Cocktail level (Figs. 4 and 5, Table 1).

Hsp27 levels in the gastrocnemius muscle increased with aging ($p < 0.001$, Fig. 6A, Table 1). No changes were detected for Hsp70 levels (Fig. 6B, Table 1). Univariate analysis further showed that both strain and age had a marked impact on HSF1 and HSF2 levels ($p < 0.050$, Fig. 6C and D, Table 1), with HCRs expressing higher protein levels of these transcription factors than LCRs and an increase in both proteins with aging.

There was a clear strain effect on both cyt C and PGC-1α contents in the gastrocnemius muscle with HCRs higher protein levels compared to

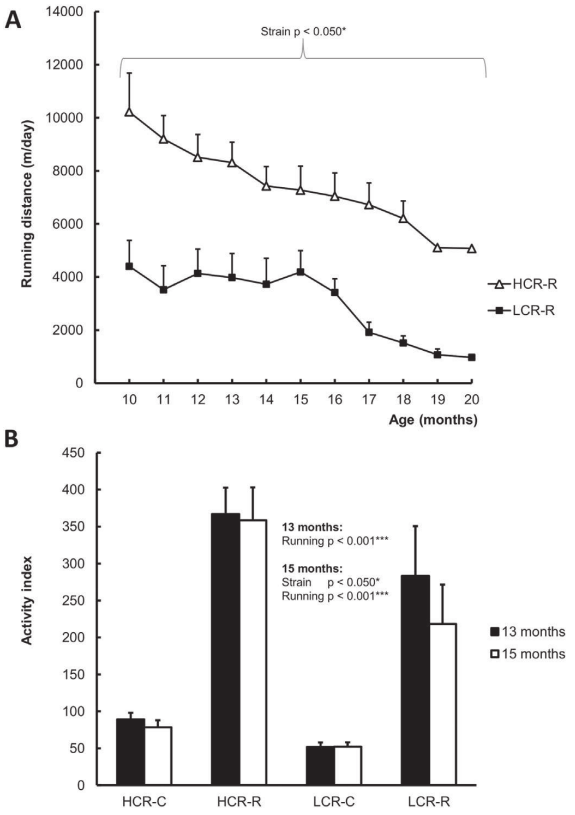

Fig. 3. Voluntary running and spontaneous activity during one-year intervention. n = 7-10/group. Values are expressed as mean ± SEM.

LCRs ($p < 0.010$, Fig. 7A and B, Table 1). Aging significantly increased PGC-1α level ($p < 0.05$, Fig. 7B, Table 1).

3.4. Citrate synthase activity

HCRs had higher citrate synthase activity compared to LCRs (strain effect $p < 0.05$). Aging or voluntary running had no significant effect on citrate synthase activity (HCR_before 2939 ± 679 vs. HCR_after 3267 ± 1081 and HCR-R_after 3554 ± 835; LCR_before 2767 ± 866 vs. LCR_after 2863 ± 917 and LCR-R_after 3545 ± 1328 [U• μg^{-1}•min^{-1}, mean ± SD]).

4. Discussion

Our results revealed that HCR rats had higher SIRT3 protein content in skeletal muscle compared to LCR rats. Also, HSF1 and HSF2 proteins showed a similar strain effect with HCRs having higher levels compared to LCRs. Aging increased the protein levels of HSF1, HSF2 and Hsp27.

The main finding from this study is that HCRs have higher SIRT3 protein content in skeletal muscle compared to LCRs (Fig. 4A, Table 1). SIRT3 in general is linked to elevated metabolism, fatty-acid oxidation and oxidative phosphorylation (Hirschey et al., 2010; Sack and Finkel, 2012), which have been established in HCRs (Gavini et al., 2014; Novak et al., 2010b; Overmyer et al., 2015). A recent study of Overmyer *et al* showed that HCRs have lower mitochondrial protein acetylation in gastrocnemius muscle during rest and exercise than LCRs; however, they did not find a difference in the SIRT3 level (Overmyer et al., 2015). Since the rats utilized in Overmyer's study were three month old male rats, whereas our rats were nine month old females, it is possible that the age and gender difference may contribute to the different results. Nonetheless, SIRT3 has been proposed to be the most prominent deacetylase in the mitochondria (Lombard et al., 2007), and Overmyer *et al* suggested that the clear difference seen in the acetylation level might be due to SIRT3 activation, but not protein level *per se*. The higher SIRT3 level and/or activation of HCRs may promote increased deacetylation in mitochondria, thereby increasing the activity of enzymes of oxidative pathways. Thus, compared to LCRs, HCRs are able to more efficiently continue ATP production through oxidative phosphorylation especially during exercise (Overmyer et al., 2015).

Furthermore, in a previous study with humans, the strongest association between sirtuins and aging has been found in the SIRT3 gene polymorphism in mitochondria (Rose et al., 2003). Rose *et al* searched for variability in the evolutionary conserved domain of the SIRT3 gene and identified a silent G/T transversion at the position 477 of the coding region (G477T polymorphism). They found that the TT genotype increased survival in the elderly even though this variant does not alter the amino acid sequence (Rose et al., 2003). In our study, we found that HCRs, that are shown to have longer lifespan than LCRs, expressed higher levels of SIRT3 protein, indicating that not only the SIRT3 variant but also the protein level may be linked to a longer lifespan.

In addition to SIRT3, the muscle tissue level of its target protein Hsp10 (after normalization to GAPDH) was higher in HCRs than in LCRs (strain effect, $p < 0.010$, *data not shown*). Though originally identified as a mitochondrial chaperone, Hsp10 is also known to be present in cytosol, cell surface, extracellular space and peripheral blood and to have various other cellular functions (Jia et al., 2011; Sadacharan et al., 2001). The ability of HSP10 to change its location is believed to be related to the protection of cells from various kinds of stressors such as infections and inflammation that are potential threats for healthy aging (Jia et al., 2011). It may be speculated, that cellular Hsp10 shares a role in healthy aging beside SIRT3.

Unlike what we hypothesized, there were no differences in the protein levels of other studied sirtuins than SIRT3 between the rat strains. In previous studies SIRT1 deacetylase activity has been shown to be similar in non-trained and trained HCR and LCR rats when the training consisted of 12 week treadmill running, whereas SIRT4 level was decreased with exercise in gastrocnemius muscle (Hart et al., 2013a, 2013b). It was speculated, that decreased SIRT4 level resulted in increased free fatty acid utilization. However, in these studies the results of HCR and LCR rats were reported separately, so there was no comparison between the rat strains on the studied sirtuins. SIRT1 is known to deacetylate PGC-1α, which links SIRT1 to oxidative metabolism (Guarente, 2013). Indeed, the activity of SIRT1, but not its expression, has been observed to regulate mitochondrial biogenesis both in human and rat skeletal muscle (Gurd et al., 2011; Koltai et al., 2010). Another study found that acute endurance exercise increases the levels of SIRT1 and PGC-1α in rat skeletal muscle (Suwa et al., 2008). In our study, we did not detect an increase in SIRT1, either caused by aging or long-term voluntary running, but HCRs displayed higher PGC-1α and cyt C levels than LCRs (strain effect, $p < 0.010$, Fig. 7). Our results from elevated levels of PGC-1α, cyt C and citrate synthase activity in HCRs skeletal muscle are consistent with previous findings of increased mitochondrial copy number, protein expression of mitochondrial respiratory complexes and citrate synthase activity of HCRs in soleus and EDL muscles compared to LCRs (Stephenson et al., 2012). Since PGC-1α plays a key role in mitochondrial biogenesis and cyt C is an indicator of mitochondrial density, the elevated PGC-1α and cyt C levels may aid HCRs to maintain their high muscle oxidative capacity during aging (Handschin and Spiegelman, 2006; Booth, 1991).

Although we did not detect an aging-related effect on sirtuins, aging significantly increased Hsp27, HSF1 and HSF2 levels (Table 1, Fig. 6). In non-stressed cells, the proteins of many Hsps are hardly detectable and they increase dramatically in response to acute stress. Once induced, Hsps directly modulate the execution of the apoptotic signaling

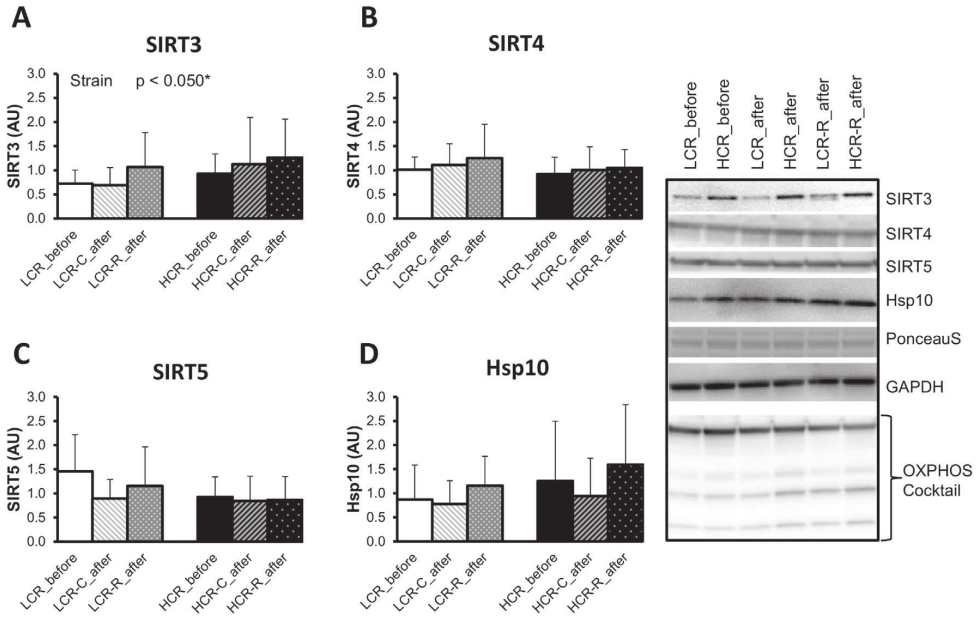

Fig. 4. Protein levels of mitochondrial sirtuins SIRT3, SIRT4 and SIRT5 and Hsp10 in gastrocnemius muscle. n = 9-10/group. Sirtuin protein and OXPHOS Cocktail levels were first normalized to corresponding Ponceau S stained actin band after which SIRT3, SIRT4 and SIRT5 were normalized to corresponding OXPHOS Cocktail level. Hsp10 was first normalized to GAPDH level and thereafter to corresponding PonceauS normalized OXPHOS Cocktail level. Values are expressed as arbitrary units (AU) mean ± SD.

pathway (Jakob et al., 1993; Schmitt et al., 2007). Thus, Hsps have a cytoprotective function: they allow the cells to adapt to gradual changes in their environment and to survive under otherwise lethal conditions (Schmitt et al., 2007). Aging in general is linked to accumulation of oxidative stress (Wei and Lee, 2002), and it appears also to attenuate the heat shock response in the myocardium of old animals (Demirel et al., 2003; Locke and Tanguay, 1996). However, in skeletal muscle, old animals seem to retain their capability to accumulate Hsps when subjected to heat shock (Locke, 2000). HSF1 and HSF2 are important for protecting cells from protein-damaging stress associated with misfolded proteins and aging as well as aging-related diseases (Rodgers et al., 2005; Westerheide et al., 2009). Interestingly, Hsp27 has been shown to increase the cellular anti-oxidant defense (Mehlen et al., 1996). Elevated HSF1, HSF2 and Hsp27 levels reported here may indicate that muscle cells of aged animals respond to aging-related accumulation of oxidative stress and attempt to compensate the stress by increasing the amount of components required for the protective molecular machinery (Murlasits et al., 2006). Moreover, aging increased the level of PGC-1α and had a tendency to increase cyt C level (Fig. 7, Table 1), not showing the expected aging-related decrease of mitochondrial function and content that has been established previously (Conley et al., 2000; Johnson et al., 2013). One reason for these unexpected results may be the fact that aging-related loss of muscle fibers is associated with a selective atrophy of type 2 (mainly glycolytic) muscle fibers, resulting in higher relative amount of type 1 (oxidative) fibers in aged muscle (Thompson, 1994; Lexell, 1995). Hence aging-related changes in skeletal muscle, especially ones in relation to oxidative capacity, are controversially reported in the literature (Houmard et al., 1998). It may be speculated that the shift in fiber-type ratio is also affecting our results. Furthermore, it is possible, that there would have been an aging effect in mitochondrial markers if we had chosen a later time point.

Table 1
Univariate analysis of strain, age and running effects of the studied protein levels.

	Variance analysis p		
Protein	Strain	Age	Running
SIRT1	0.412	0.326	0.727
SIRT2	0.232	0.825	0.677
SIRT3	0.020*	0.712	0.906
SIRT4	0.766	0.591	0.952
SIRT5	0.191	0.573	0.385
SIRT6	0.691	0.739	0.969
SIRT7	0.328	0.494	0.582
Hsp10	0.169	0.288	0.933
Hsp27	0.141	<0.001***	0.381
Hsp70	0.454	0.316	0.091
HSF1	<0.001***	0.003**	0.274
HSF2	0.005**	0.020*	0.114
PGC-1α	0.003**	0.049*	0.108
Cyt C	<0.001***	0.087	0.187

Our second hypothesis was that the exercise response to the studied protein levels would be more profound in HCRs than in LCRs, due to their higher running activity. As expected, HCRs did run significantly more than LCRs (Fig. 3A). Surprisingly, voluntary running had no significant effect on the studied proteins. It has been established in previous studies, that endurance training increases PGC-1α and cyt C levels as well as mitochondrial content and respiratory capacity in skeletal muscle (Holloszy and Coyle, 1984). It seems that in gastrocnemius muscle, there was no pressure to increase the oxidative capacity to significant extent, at least not at the very late time point chosen in our study. PGC-1α is known to respond to exercise acutely (Baar et al., 2002), so as in our study the running distance decreased gradually over time during the one year intervention (Fig. 3A), also the pressure for change decreased. It is possible that we would have seen an adaptive increase in the studied mitochondrial markers if we had chosen an earlier time point or used other muscle instead of gastrocnemius to measure these parameters.

Our data showed that high intrinsic aerobic capacity is linked to high SIRT3 protein levels in skeletal muscle. According to our former study at the age of 21 months all the HCRs in the control group (HCR-C) were

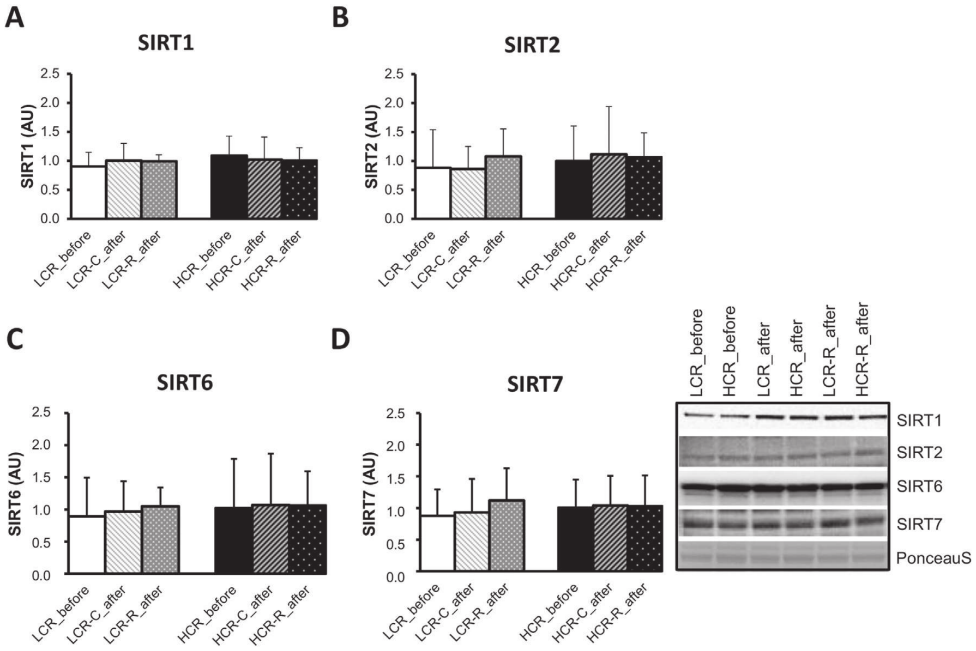

Fig. 5. Protein levels of nuclear (SIRT1, SIRT6 and SIRT7) and both nuclear and cytoplasmic (SIRT2) sirtuins in gastrocnemius muscle. n = 9-10/group. Values are expressed as arbitrary units (AU) mean ± SD.

surviving, whereas the survivability percent for the other studied groups were the following: 95% for HCR-R, 90% for LCR-C and 65% for LCR-R group (Karvinen et al., 2015). In our cross-sectional study we had same number of samples from both rat strains at time point 21 months randomly selected from a larger group size to compare the differences at muscular level. However, we did not detect any differences in the protein levels of the other studied sirtuins between HCR and LCR rats with aging or voluntary exercise. We were not able to measure

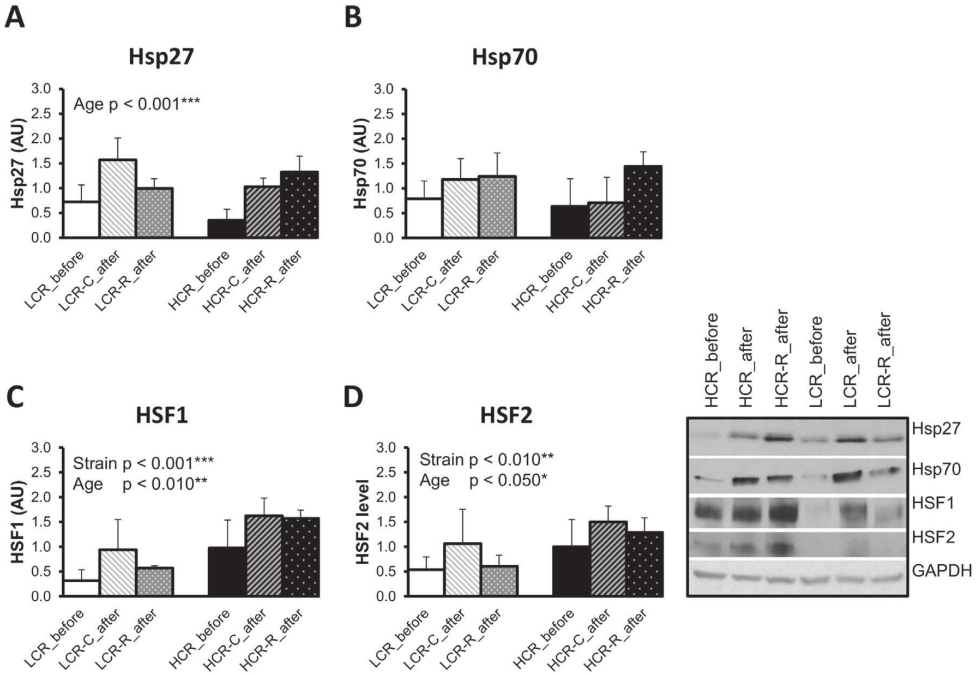

Fig. 6. Hsp and HSF protein levels in gastrocnemius muscle. n = 4-5/group. Values are expressed as arbitrary units (AU) mean ± SD.

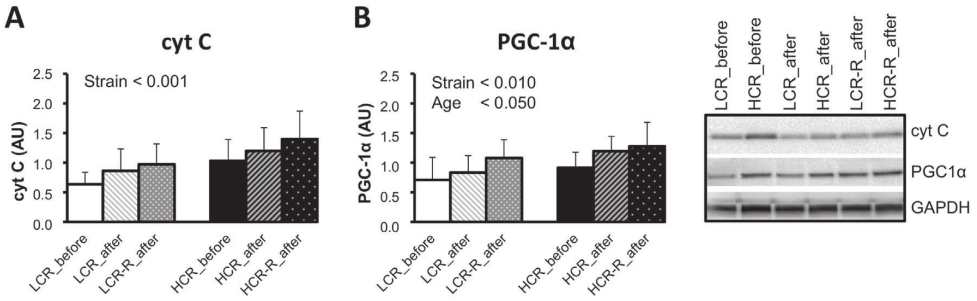

Fig. 7. Cyt C and PGC-1α protein levels in gastrocnemius muscle. n = 9-10/group. Values are expressed as arbitrary units (AU) mean ± SD.

the activities of sirtuins, and thus, it is possible that there are age-related or exercise-induced differences in the activities of the proteins studied as well as other proteins that we were not able to detect with the methods utilized in this study. Since HSF1, HSF2 and Hsp27 levels increased with aging in both rat strains, our study suggests that aged muscles remain responsive to stress and can compensate for aging-related accumulative oxidative stress by increasing the amount of protective molecular machinery. To the best of our knowledge, this study provides the first evidence for a direct link between elevated SIRT3 protein levels and intrinsic aerobic capacity, which may be associated with an extended lifespan in mammals.

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References

Baar, K., Wende, A.R., Jones, T.E., Marison, M., Nolte, L.A., Chen, M., et al., 2002. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J. 16 (14), 1879–1886 Dec.
Beckmann, R.P., Mizzen, L.E., Welch, W.J., 1990. Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. Science 248 (4957), 850–854 May 18.
Biesiadecki, B.J., Brand, P.H., Koch, L.G., Britton, S.L., 1999. A gravimetric method for the measurement of total spontaneous activity in rats. Proc. Soc. Exp. Biol. Med. 222 (1), 65–69 Oct.
Bishop, N.A., Guarente, L., 2007. Genetic links between diet and lifespan: shared mechanisms from yeast to humans. Nat. Rev. Genet. 8 (11), 835–844 Nov.
Bodkin, N.L., Alexander, T.M., Ortmeyer, H.K., Johnson, E., Hansen, B.C., 2003. Mortality and morbidity in laboratory-maintained Rhesus monkeys and effects of long-term dietary restriction. J. Gerontol. A Biol. Sci. Med. Sci. 58 (3), 212–219 Mar.
Booth, F.W., 1991. Cytochrome c protein synthesis rate in rat skeletal muscle. J. Appl. Physiol. (1985) 71 (4), 1225–1230 Oct.
Bordone, L., Cohen, D., Robinson, A., Motta, M.C., van Veen, E., Czopik, A., et al., 2007. SIRT1 transgenic mice show phenotypes resembling calorie restriction. Aging Cell 6 (6), 759–767 Dec.
Conley, K.E., Jubrias, S.A., Esselman, P.C., 2000. Oxidative capacity and ageing in human muscle. J. Physiol. 526 (Pt 1), 203–210 Jul 1.
Demirel, H.A., Hamilton, K.L., Shanely, R.A., Tumer, N., Koroly, M.J., Powers, S.K., 2003. Age and attenuation of exercise-induced myocardial HSP72 accumulation. Am. J. Physiol. Heart Circ. Physiol. 285 (4), H1609–H1615 Oct.
Ferrara, N., Rinaldi, B., Corbi, G., Conti, V., Stiuso, P., Boccuti, S., et al., 2008. Exercise training promotes SIRT1 activity in aged rats. Rejuvenation Res. 11 (1), 139–150 Feb.
Finkel, T., Deng, C.X., Mostoslavsky, R., 2009. Recent progress in the biology and physiology of sirtuins. Nature 460 (7255), 587–591 Jul 30.
Gavini, C.K., Mukherjee, S., Shukla, C., Britton, S.L., Koch, L.G., Shi, H., et al., 2014. Leanness and heightened nonresting energy expenditure: role of skeletal muscle activity thermogenesis. Am. J. Physiol. Endocrinol. Metab. 306 (6), E635–E647 Mar.
Guarente, L., 2013. Introduction: sirtuins in aging and diseases. Methods Mol. Biol. 1077, 3–10.
Gurd, B.J., Yoshida, Y., McFarlan, J.T., Holloway, G.P., Moyes, C.D., Heigenhauser, G.J., et al., 2011. Nuclear SIRT1 activity, but not protein content, regulates mitochondrial biogenesis in rat and human skeletal muscle. Am. J. Phys. Regul. Integr. Comp. Phys. 301 (1), R67–R75 Jul.
Gurd, B.J., Holloway, G.P., Yoshida, Y., Bonen, A., 2012y. In mammalian muscle, SIRT3 is present in mitochondria and not in the nucleus; and SIRT3 is upregulated by chronic muscle contraction in an adenosine monophosphate-activated protein kinase-independent manner. Metabolism 61 (5), 733–741 May.
Handschin, C., Spiegelman, B.M., 2006. Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. Endocr. Rev. 27 (7), 728–735 Dec.
Hart, N., Sarga, L., Csende, Z., Koch, L.G., Britton, S.L., Davies, K.J., et al., 2013a. Resveratrol attenuates exercise-induced adaptive responses in rats selectively bred for low running performance. Dose-Response 12 (1), 57–71 Jun 4.
Hart, N., Sarga, L., Csende, Z., Koltai, E., Koch, L.G., Britton, S.L., et al., 2013b. Resveratrol enhances exercise training responses in rats selectively bred for high running performance. Food Chem. Toxicol. 61, 53–59 Nov.
Hirschey, M.D., Shimazu, T., Goetzman, E., Jing, E., Schwer, B., Lombard, D.B., et al., 2010. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature 464 (7285), 121–125 Mar 4.
Ho, L., Titus, A.S., Banerjee, K.K., George, S., Lin, W., Deota, S., et al., 2013. SIRT4 regulates ATP homeostasis and mediates a retrograde signaling via AMPK. Aging (Albany NY) 5 (11), 835–849 Nov.
Holloszy, J.O., Coyle, E.F., 1984. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. J. Appl. Physiol. 56 (4), 831–838 Apr.
Hoppeler, H., 1986. Exercise-induced ultrastructural changes in skeletal muscle. Int. J. Sports Med. 7 (4), 187–204 Aug.
Houmard, J.A., Weidner, M.L., Gavigan, K.E., Tyndall, G.L., Hickey, M.S., Alshami, A., 1998. Fiber type and citrate synthase activity in the human gastrocnemius and vastus lateralis with aging. J. Appl. Physiol. (1985) 85 (4), 1337–1341 Oct.
Jakob, U., Gaestel, M., Engel, K., Buchner, J., 1993. Small heat shock proteins are molecular chaperones. J. Biol. Chem. 268 (3), 1517–1520 Jan 25.
Jia, H., Halilou, A.I., Hu, L., Cai, W., Liu, J., Huang, B., 2011. Heat shock protein 10 (Hsp10) in immune-related diseases: one coin, two sides. Int. J. Biochem. Mol. Biol. 2 (1), 47–57.
Jia, G., Su, L., Singhal, S., Liu, X., 2012. Emerging roles of SIRT6 on telomere maintenance, DNA repair, metabolism and mammalian aging. Mol. Cell. Biochem. 364 (1-2), 345–350 May.
Johnson, M.L., Robinson, M.M., Nair, K.S., 2013. Skeletal muscle aging and the mitochondrion. Trends Endocrinol. Metab. 24 (5), 247–256 May.
Kaeberlein, M., McVey, M., Guarente, L., 1999. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev. 13 (19), 2570–2580 Oct 1.
Kampinga, H.H., Hageman, J., Vos, M.J., Kubota, H., Tanguay, R.M., Bruford, E.A., et al., 2009. Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones 14 (1), 105–111 Jan.
Kanfi, Y., Naiman, S., Amir, G., Peshti, V., Zinman, G., Nahum, L., et al., 2012. The sirtuin SIRT6 regulates lifespan in male mice. Nature 483 (7388), 218–221 Feb 22.
Karvinen, S., Waller, K., Silvennoinen, M., Koch, L.G., Britton, S.L., Kaprio, J., et al., 2015. Physical activity in adulthood: genes and mortality. Sci. Rep. 5, 18259 Dec 15.
Koch, L.G., Britton, S.L., 2001. Artificial selection for intrinsic aerobic endurance running capacity in rats. Physiol. Genomics 5 (1), 45–52 Feb 7.
Koch, L.G., Kemi, O.J., Qi, N., Leng, S.X., Bijma, P., Gilligan, L.J., et al., 2011. Intrinsic Aerobic Capacity Sets a Divide for Aging and Longevity. Circ. Res. Sep 15.

Koltai, E., Szabo, Z., Atalay, M., Boldogh, I., Naito, H., Goto, S., et al., 2010. Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats. Mech. Ageing Dev. 131 (1), 21–28 Jan.
Kruk, P.A., Rampino, N.J., Bohr, V.A., 1995. DNA damage and repair in telomeres: relation to aging. Proc. Natl. Acad. Sci. U. S. A. 92 (1), 258–262 Jan 3.
Lexell, J., 1995. Human aging, muscle mass, and fiber type composition. J. Gerontol. A Biol. Sci. Med. Sci. 50 Nov. Spec No:11-6.
Liu, D.J., Hammer, D., Komlos, D., Chen, K.Y., Firestein, B.L., Liu, A.Y., 2014. SIRT1 knockdown promotes neural differentiation and attenuates the heat shock response. J. Cell. Physiol. 229 (9), 1224–1235 Sep.
Locke, M., 2000. Heat shock transcription factor activation and hsp72 accumulation in aged skeletal muscle. Cell Stress Chaperones 5 (1), 45–51 Jan.
Locke, M., Tanguay, R.M., 1996. Diminished heat shock response in the aged myocardium. Cell Stress Chaperones 1 (4), 251–260 Dec.
Lollgen, H., Bockenhoff, A., Knapp, G., 2009. Physical activity and all-cause mortality: an updated meta-analysis with different intensity categories. Int. J. Sports Med. 30 (3), 213–224 Mar.
Lombard, D.B., Alt, F.W., Cheng, H.L., Bunkenborg, J., Streeper, R.S., Mostoslavsky, R., et al., 2007. Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. Mol. Cell. Biol. 27 (24), 8807–8814 Dec.
Lu, Z., Chen, Y., Aponte, A.M., Battaglia, V., Gucek, M., Sack, M.N., 2015. Prolonged fasting identifies heat shock protein 10 as a Sirtuin 3 substrate: elucidating a new mechanism linking mitochondrial protein acetylation to fatty acid oxidation enzyme folding and function. J. Biol. Chem. 290 (4), 2466–2476 Jan 23.
McBurney, M.W., Yang, X., Jardine, K., Hixon, M., Boekelheide, K., Webb, J.R., et al., 2003. The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. Mol. Cell. Biol. 23 (1), 38–54 Jan.
Mehlen, P., Kretz-Remy, C., Preville, X., Arrigo, A.P., 1996. Human hsp27, Drosophila hsp27 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFalpha-induced cell death. EMBO J. 15 (11), 2695–2706 Jun 3.
Michishita, E., McCord, R.A., Berber, E., Kioi, M., Padilla-Nash, H., Damian, M., et al., 2008. SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. Nature 452 (7186), 492–496 Mar 27.
Mostoslavsky, R., Chua, K.F., Lombard, D.B., Pang, W.W., Fischer, M.R., Gellon, L., et al., 2006. Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. Cell 124 (2), 315–329 Jan 27.
Murlasits, Z., Cutlip, R.G., Geronilla, K.B., Rao, K.M., Wonderlin, W.F., Alway, S.E., 2006. Resistance training increases heat shock protein levels in skeletal muscle of young and old rats. Exp. Gerontol. 41 (4), 398–406 Apr.
Myers, J., Prakash, M., Froelicher, V., Do, D., Partington, S., Atwood, J.E., 2002. Exercise capacity and mortality among men referred for exercise testing. N. Engl. J. Med. 346 (11), 793–801 Mar 14.
Nakagawa, T., Guarente, L., 2011. Sirtuins at a glance. J. Cell Sci. 124 (Pt 6), 833–838 Mar 15.
Nakagawa, T., Lomb, D.J., Haigis, M.C., Guarente, L., 2009. SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. Cell 137 (3), 560–570 May 1.
Nasrin, N., Wu, X., Fortier, E., Feng, Y., 2010. Bare' OC, Chen S, et al. SIRT4 regulates fatty acid oxidation and mitochondrial gene expression in liver and muscle cells. J. Biol. Chem. 285 (42), 31995–32002 Oct 15.
Novak, C.M., Escande, C., Burghardt, P.R., Zhang, M., Barbosa, M.T., Chini, E.N., et al., 2010a. Spontaneous activity, economy of activity, and resistance to diet-induced obesity in rats bred for high intrinsic aerobic capacity. Horm. Behav. 58 (3), 355–367 Aug.
Novak, C.M., Escande, C., Burghardt, P.R., Zhang, M., Barbosa, M.T., Chini, E.N., et al., 2010b. Spontaneous activity, economy of activity, and resistance to diet-induced obesity in rats bred for high intrinsic aerobic capacity. Horm. Behav. 58 (3), 355–367 Aug.
Olovnikov, A.M., 1996. Telomeres, telomerase, and aging: origin of the theory. Exp. Gerontol. 31 (4), 443–448 Jul-Aug.
Overmyer, K.A., Evans, C.R., Qi, N.R., Minogue, C.E., Carson, J.J., Chermside-Scabbo, C.J., et al., 2015. Maximal oxidative capacity during exercise is associated with skeletal muscle fuel selection and dynamic changes in mitochondrial protein acetylation. Cell Metab. 21 (3), 468–478 Mar 3.
Palacios, O.M., Carmona, J.J., Michan, S., Chen, K.Y., Manabe, Y., Ward III, J.L., et al., 2009. Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle. Aging (Albany NY) 1 (9), 771–783 Aug 15.
Park, S.H., Zhu, Y., Ozden, O., Kim, H.S., Jiang, H., Deng, C.X., et al., 2012. SIRT2 is a tumor suppressor that connects aging, acetylome, cell cycle signaling, and carcinogenesis. Transl. Cancer Res. 1 (1), 15–21 Jun 1.
Park, J., Chen, Y., Tishkoff, D.X., Peng, C., Tan, M., Dai, L., et al., 2013. SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways. Mol. Cell 50 (6), 919–930 Jun 27.
Rodgers, J.T., Lerin, C., Haas, W., Gygi, S.P., Spiegelman, B.M., Puigserver, P., 2005. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature 434 (7029), 113–118 Mar 3.
Rogina, B., Helfand, S.L., 2004. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. Proc. Natl. Acad. Sci. U. S. A. 101 (45), 15998–16003 Nov 9.
Rose, G., Dato, S., Altomare, K., Bellizzi, D., Garasto, S., Greco, V., et al., 2003. Variability of the SIRT3 gene, human silent information regulator Sir2 homologue, and survivorship in the elderly. Exp. Gerontol. 38 (10), 1065–1070 Oct.
Sack, M.N., Finkel, T., 2012. Mitochondrial metabolism, sirtuins, and aging. Cold Spring Harb. Perspect. Biol. 4 (12). http://dx.doi.org/10.1101/cshperspect.a013102 Dec 1.
Sadacharan, S.K., Cavanagh, A.C., Gupta, R.S., 2001. Immunoelectron microscopy provides evidence for the presence of mitochondrial heat shock 10-kDa protein (chaperonin 10) in red blood cells and a variety of secretory granules. Histochem. Cell Biol. 116 (6), 507–517 Dec.
Satoh, A., Brace, C.S., Rensing, N., Cliften, P., Wozniak, D.F., Herzog, E.D., et al., 2013. Sirt1 extends life span and delays aging in mice through the regulation of Nk2 homeobox 1 in the DMH and LH. Cell Metab. 18 (3), 416–430 Sep 3.
Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G., Garrido, C., 2007n. Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. J. Leukoc. Biol. 81 (1), 15–27 Jan.
Silvennoinen, M., Rantalainen, T., Kainulainen, H., 2014. Validation of a method to measure total spontaneous physical activity of sedentary and voluntary running mice. J. Neurosci. Methods 235 (0), 51–58 9/30.
Stephenson, E.J., Stepto, N.K., Koch, L.G., Britton, S.L., Hawley, J.A., 2012. Divergent skeletal muscle respiratory capacities in rats artificially selected for high and low running ability: a role for Nor1? J. Appl. Physiol. (1985) 113 (9), 1403–1412 Nov.
Suwa, M., Nakano, H., Radak, Z., Kumagai, S., 2008. Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor gamma coactivator-1alpha protein expressions in rat skeletal muscle. Metabolism 57 (7), 986–998 Jul.
Thompson, L.V., 1994. Effects of age and training on skeletal muscle physiology and performance. Phys. Ther. 74 (1), 71–81 Jan.
Vakhrusheva, O., Smolka, C., Gajawada, P., Kostin, S., Boettger, T., Kubin, T., et al., 2008. Sirt7 increases stress resistance of cardiomyocytes and prevents apoptosis and inflammatory cardiomyopathy in mice. Circ. Res. 102 (6), 703–710 Mar 28.
Van Remmen, H., Richardson, A., 2001. Oxidative damage to mitochondria and aging. Exp. Gerontol. 36 (7), 957–968 Jul.
Verdin, E., Hirschey, M.D., Finley, L.W., Haigis, M.C., 2010. Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling. Trends Biochem. Sci. 35 (12), 669–675 Dec.
Vihervaara, A., Sergelius, C., Vasara, J., Blom, M.A., Elsing, A.N., Roos-Mattjus, P., et al., 2013. Transcriptional response to stress in the dynamic chromatin environment of cycling and mitotic cells. Proc. Natl. Acad. Sci. U. S. A. 110 (36), E3388–E3397 Sep 3.
Waters, R.P., Renner, K.J., Pringle, R.B., Summers, C.H., Britton, S.L., Koch, L.G., et al., 2008. Selection for aerobic capacity affects corticosterone, monoamines and wheel-running activity. Physiol. Behav. 93 (4-5), 1044–1054 Mar 18.
Wei, Y.H., Lee, H.C., 2002. Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. Exp. Biol. Med. (Maywood) 227 (9), 671–682 Oct.
Westerheide, S.D., Anckar, J., Stevens Jr., S.M., Sistonen, L., Morimoto, R.I., 2009. Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. Science 323 (5917), 1063–1066 Feb 20.
Xiao, X., Zuo, X., Davis, A.A., McMillan, D.R., Curry, B.B., Richardson, J.A., et al., 1999. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. EMBO J. 18 (21), 5943–5952 Nov 1.
Xiao, C., Kim, H.S., Lahusen, T., Wang, R.H., Xu, X., Gavrilova, O., et al., 2010. SIRT6 deficiency results in severe hypoglycemia by enhancing both basal and insulin-stimulated glucose uptake in mice. J. Biol. Chem. 285 (47), 36776–36784 Nov 19.

IV

VOLUNTARY RUNNING AIDS TO MAINTAIN HIGH THERMOGENESIS IN RATS BRED FOR HIGH AEROBIC CAPACITY

by

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Voluntary Running Aids to Maintain High Thermogenesis in Rats Bred for High Aerobic Capacity

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Abstract

Body heat generation, i.e. thermogenesis, is a significant component of the metabolic rate, which in turn affects weight gain and health. Thermogenesis is linked to physical activity (PA) level. However, it is not known how genetic background, aging, and voluntary running affect thermogenesis. Here we study the association of intrinsic aerobic capacity, PA, aging and blood glucose concentration with thermogenesis. For this study ten high capacity runner (HCR) and ten low capacity runner (LCR) female rats were utilized. The body temperature of HCR and LCR rats was measured at a normal stage and after one year voluntary running/control intervention to explore the effects of aging and PA. Also, it was studied how injected glucose or placebo (physiological saline solution) and spontaneous activity affect the body temperature. HCRs had on average 1.3°C. higher body temperature than LCRs ($p < 0.001$). Aging decreased the body temperature level of HCRs into similar levels with LCRs. The opportunity to run voluntarily had a marked impact on the body temperature of HCRs ($p < 0.001$) aiding them to maintain body temperature at similar level as at younger age. Compared to LCRs, HCRs were spontaneously more active, had higher relative gastrocnemius muscle mass and higher UCP2, PGC-1α, cyt c and OXPHOS contents in the skeletal muscle ($p < 0.050$). These results suggest that higher PA level together with greater relative muscle mass and higher mitochondrial content/function contribute to the more efficient thermogenesis of HCRs. Neither aging nor voluntary training had a significant impact on body temperature of LCRs. Glucose injection lowered the body temperature of LCRs ($p < 0.050$), but not that of HCRs. To conclude, HCRs had higher body temperature at younger age compared to LCRs at untrained state. Voluntary running aided HCRs to maintain high thermogenesis during aging, which states that high PA level was crucial in maintaining the high body temperature of HCRs.

Keywords: Thermogenesis, body temperature, physical activity, aerobic capacity, aging, skeletal muscle, sirtuins

1 Introduction

Thermogenesis is an energy demanding process that has a significant contribution to daily total energy expenditure (TEE), body weight and health (Levine, Eberhardt & Jensen 1999, Rosenbaum et al. 2008). High thermogenesis is linked to high physical activity (PA) level, as body temperature rises as a result of increased muscular activity (Gleeson 1998). In rodents it has been established, that high PA in turn is tightly associated with high aerobic capacity (Novak et al. 2010). Whether the level of thermogenesis is due to inborn aerobic capacity or a consequence of PA level *per se* remains unclear. Furthermore, it is not known how genetic background, aging and voluntary running affect thermogenesis. Here we study the effects of intrinsic aerobic capacity, voluntary running, aging, and blood glucose concentration on thermogenesis.

Several features affect thermogenesis and TEE, such as age, physical activity, meals (processing nutrients), stress, and in females the stage of estrous cycle (Horan et al. 1988, Kent, Hurd & Satinoff 1991, Kontani et al. 2002, Yamashita et al. 1994, Waters et al. 2010, Landsberg, Saville & Young 1984). Physical activity, whether it is endurance or strength training, or just normal daily activities, correlates positively with body temperature (Nozu et al. 1992, Tonkonogi et al. 2000). Thermic effect of food is known to increase energy expenditure and thermogenesis (Rothwell & Stock 1979, Cannon & Nedergaard 2004), whereas stress causes an increase both in blood glucose concentration and body temperature via stress hormones and stress related behaviour (Vachon & Moreau 2001, Blanchard, Griebel & Blanchard 2001). In females, the stage of estrous cycle is known to affect the body temperature; temperature increases during proestrus, when progesterone and estrogen concentrations are highest, while during estrus there is a drop in body temperature (Kent, Hurd & Satinoff 1991, Marrone, Gentry & Wade 1976).

At cellular level, the amount and efficiency of mitochondria and uncoupling proteins (UCP) play the main role in thermogenesis (Rousset et al. 2004, Cannon & Nedergaard 2004). UCP1 is primarily found in the mitochondria of brown adipose tissue (BAT), whereas UCP3 is the main uncoupling protein expressed in skeletal muscle (Rousset et al. 2004). UCP2 is a mitochondrial uncoupling protein that separates oxidative phosphorylation from ATP synthesis with energy dissipated as heat. Although UCP3 and UCP2 share similar features, UCP2 is mainly thought to control the production of mitochondria-derived reactive oxygen species, whereas UCP3 plays a main role in non-shivering thermogenesis (Boss et al. 1997, Rousset et al. 2004). Besides UCPs, also amount and efficiency of mitochondria to produce heat affects body temperature. PGC-1α plays a key role in mitochondrial biogenesis whereas cytochrome c (cyt c) is a crucial component of the electron transport chain in mitochondria and hence these proteins are essential for muscle oxidative capacity (Huttemann et al. 2011, Lin et al. 2015). Endurance exercise is known to increase PGC-1α levels and mitochondrial biogenesis in muscle, although UCP levels are found to be reduced (Jones et al. 2003, Freyssenet, Berthon & Denis 1996, Holloszy 2008, Nozu et al. 1992, Pilegaard et al. 2000, Russell et al. 2003). Aging in general causes decrease in thermogenesis via lower UCP levels, reduced response to noradrenaline and diminished BAT content (Kontani et al. 2002, Horan et al. 1988, Yamashita et al. 1994).

BAT has for long been considered as the main thermogenic organ (van den Berg et al. 2011). At present, in addition to BAT, skeletal muscle has been demonstrated to significantly contribute to thermogenesis (Gavini et al. 2014). Since approximately 40% of total body mass is composed of muscle, the capacity of skeletal muscle to contribute to whole-body

energy expenditure is reasonable. The contribution of skeletal muscle mitochondrial uncoupling to weight gain has been demonstrated in several mouse models (Son et al. 2004, Costford et al. 2008, Choi et al. 2007). It has been shown that UCP3 overexpression protects mice from high-fat diet induced insulin resistance and obesity (Choi et al. 2007, Clapham et al. 2000). Recent studies from genetically contrasting rat lines reveal that intrinsic aerobic capacity has a major role in TEE, physical activity level and non-exercise activity thermogenesis (NEAT) (Gavini et al. 2014, Novak & Levine 2007). Rats selectively bred for high aerobic endurance capacity (HCR = high-capacity runner) are also spontaneously more active and consume more energy compared to their counterparts that are selectively bred for low aerobic endurance capacity (LCR = low-capacity runner) (Kivelä et al. 2010, Koch & Britton 2001). Furthermore, HCRs have lower risk to become obese and have longer lifespan compared to LCRs, whereas LCRs are prone to gain excess body weight and develop metabolic disorders (Kivelä et al. 2010, Noland et al. 2007, Koch et al. 2011, Wisloff et al. 2005).

Body temperature is tightly associated with metabolic rate (Landsberg 2012, Geiser 1988, Heikens et al. 2011); in fact one degree C rise in temperature is associated with a 10–13% increment in oxygen consumption (Landsberg 2012). The elevation in temperature itself is responsible for speeding up metabolism, since enzyme-catalyzed reactions are enhanced in higher temperatures (Landsberg et al. 2009). We recently noticed that besides the differences between the HCR and LCR rat lines listed above, anesthetized HCRs seemed to have higher body temperature compared to LCRs. We wanted to study this novel finding more carefully and measured the body temperature of HCR and LCR rats at normal stage and studied the effects of aging and voluntary running for one year. We also followed the body temperature during glucose tolerance and placebo test to see the effect of stress (injection), high blood glucose and spontaneous activity on body temperature. Our first hypothesis was that HCR rats have intrinsically higher body temperature compared to LCRs due to their higher mitochondrial capacity to produce heat. Secondly, we hypothesized that aging would decrease body temperature levels due to aging related loss of mitochondrial function and decreased expression of the UCP in skeletal muscle and BAT, but the difference between the HCR/LCR rat lines would still be evident. Thirdly, we assumed that voluntary running would increase the body temperature in both rat lines due to increased mitochondrial biogenesis, thus aiding to prevent age related decrease in mitochondrial function. Our final hypothesis was that glucose injection would increase the body temperature especially in the energy-dissipating HCRs, since BAT as insulin sensitive tissue may have a major role in dissipating extra energy as heat (Stanford et al. 2013, Cannon & Nedergaard 2004).

2 Materials and Methods

2.1 Animal lines

The HCR/LCR contrasting rat model was produced via two-way artificial selection, starting from a founder population of 186 genetically heterogeneous rats (N:NIH stock), as described previously (Koch & Britton 2001). Endurance running capacity was assessed at the University of Michigan (Ann Arbor, Michigan, USA) with a speed-ramped treadmill running test (15° slope, initial velocity of 10 m•min^{-1}, increased 1 m/min every 2 min) when the rats were 11 weeks of age. For the glucose and placebo tests described here, 20 female rats (10 HCR and 10 LCR) from the 27th generation of selection were used (Fig. 1, Set 1). First non-trained animals were tested before one-year voluntary intervention (age 9 months) and the tests were repeated as follow-up after the intervention (age 21 months). We also had extra 60 female rats from the generations 23-27 of selection with the same voluntary running intervention to prepare Western blot samples from gastrocnemius muscle and BAT at the same time points (Fig. 1, Set 2). The rats lived in an environmentally controlled facility (12/12 h light-dark cycle, 22°C) and received water and standard feed (R36, Labfor, Stockholm, Sweden) *ad libitum*.

2.1.1 Body temperature: Baseline measurement

Body temperature was assessed by measuring rectal temperature (Fluke 52 k/J Thermometer) after 2 h of fasting before dividing the rats into separate intervention groups (n = 10/group). Measurement was repeated in separate days and the average of two measurements was used for the statistical analyses.

2.2 Intervention

Before the first measurements, at the age of 9 months, the rats were housed two per cage in standard cages. After the first measurements rats were divided evenly in weight and maximal running capacity matched groups (n = 5); HCR (control), HCR-R (runner), LCR (control) and LCR-R (runner) (Fig. 1, Set 1). Control rats lived in a standard cage conditions and runners had an access to running wheels that were connected to a computerized recording system to follow the running distance throughout intervention. During this one year intervention the rats were housed one per cage. The follow-up measurements were performed at the age of 21 months.

2.2.1Voluntary running distance, body weight and energy intake

Voluntary running distance in the running wheels was followed throughout the one-year intervention with a self-constructed computerized recording system (Acer Verinton 6900Pro, 32 bit processor produced by Intel, Windows XP). Total wheel laps were recorded continuously, and the total running distance per day was determined by multiplying the number of wheel rotations by the circumference of the running wheel (Ø 34.5 cm). From daily running distances an average daily running distance for every two-week period was calculated. Body weight and energy intake of the rats were followed throughout the one year intervention by weighing the rats and consumed feed every second week. Energy intake was calculated as two week feed consumption from the energy content informed by the manufacturer (Labfor).

2.2.2 Body temperature: Before and after intervention measurements

To study the effects of rat line, aging and voluntary running before and after one-year voluntary running intervention, body temperature was measured similarly to the baseline measurements described above. Measurements were done after 5 h of fasting from the same rats as used for the intervention (n = 5/group). The average of the fasting (F) point measurements from each rat from glucose tolerance and placebo tests were used for the statistical analyses.

2.2.3 Glucose tolerance and placebo tests

The glucose tolerance and placebo tests were performed twice, first at the age of 9 months and second time at the age of 21 months (Fig. 1). Tests were done in two parallel sets in each day, between 9.30 a.m. and 3.30 p.m. Rats from different groups were randomized to treatment sets to account the possible effect of circadian rhythm on blood glucose level. Two animals were excluded from the follow-up measurement of old rats due to aging symptoms, leaving the following group sizes for second measurements; HCR (n = 4), HCR-R (n = 5), LCR (n = 4) and LCR-R (n = 5). Rats were deprived of food for 5 h before measurements. The running wheels of the runner rats were blocked 5 h before the measurements disabling the movement of the wheel to avoid the possible acute effects of running on the measurements. Body weight was measured before each test. At the time point 0 either 2 g/kg of glucose (20 % solution) or equal volume of placebo (physiological saline solution) was injected into the peritoneal cavity. Rectal temperature and blood glucose were measured at time points 0, 30, 60, and 120 min after the injection. Blood samples for further analyses were collected before starting the glucose/placebo protocol (fasting sample).

2.2.3.1 Heat generation

Rectal temperature was measured at time points 0, 30, 60, and 120 min after the injection (Fluke 52 k/J Thermometer). To compare the estimate of heat generation during placebo and glucose tolerance tests the area under curve (AUC) values of rectal temperature were calculated from each group during both tests between time points 0-120 min normalized with 0 min level. AUC values were used for the statistical analyses of the effect of rat line, running and treatment on heat generation.

2.2.3.2 Spontaneous activity

Spontaneous activity of the rats was followed throughout the glucose tolerance and placebo tests with ground reaction force recording as described before (Silvennoinen, Rantalainen & Kainulainen 2014). The rat cages were placed on top of individual ground reaction force plates 30 min before the start of the experiments and removed from the plate after the last blood glucose measurement. The absolute values of the differences between consecutive force values were calculated. The mean of the absolute values were calculated from every second from total 20 values per second. To obtain activity index, a single value for total spontaneous activity, the one second means were summed for the total measurement time and the sum was divided by the body mass (kg) of the measured rat (Biesiadecki et al. 1999, Silvennoinen, Rantalainen & Kainulainen 2014). The data used in statistical analyses were presented as 30 min averages. The total spontaneous activity was also calculated during the tests as a sum of activity index from the whole test period.

2.2.3.3 Blood glucose concentration

Blood glucose was measured at time points 0, 30, 60, and 120 min after the injection (HemoCue Glucose 201 RT). AUC normalized with 0 min concentration from the placebo and glucose tests were used for the statistical analyses of the effect of rat line, running and treatment on blood glucose concentration.

2.2.3.4 Phase of estrous cycle

Phase of estrous cycle was followed one week during the glucose tolerance and placebo measurements after the intervention (age 21 months). Estrous cycle samples were collected with physiological saline solution from vagina to a pipette, stained with methylene blue (Giemsas azur-eosin; methylenblaulösung, Merck 9204) and observed under a light microscope. Each sample was categorized to one of the four menstrual cycle states: proestrus, estrus, diestrus or metestrus.

2.2.4 Tissue processing

Gastrocnemius muscle (n = 10/group) and BAT (n = 5/group) samples were collected from the Set 2 rats (Fig. 1). The snap frozen samples were homogenized in liquid nitrogen and dissolved in ice-cold buffer (20 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM, β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% NP-40, 0.2% sodium deoxycholate, and 3% protease and phosphatase inhibitor coctail (P 78443; Pierce, Rockford, IL). The muscle homogenate was thereafter centrifuged at 10 000 *g* for 10 min at 4°C. Total protein content was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) with an automated KoneLab instrument (Thermo Scientific, Vantaa, Finland).

2.2.4.1 Citrate synthase activity

Citrate synthase activity ($U \bullet \mu g^{-1} \bullet min^{-1}$) in the gastrocnemius muscle (n = 10/group) was measured from the same muscle homogenates that were used to determine the total protein content (Citrate Synthase Assay Kit Sigma-Aldrich) with an automated KoneLab instrument (Thermo Scientific).

2.2.4.2 Western blot analyses

Aliquots of muscle homogenate were solubilized in Laemmli sample buffer and heated at 95°C to denaturate proteins, except for Total OXPHOS Cocktail, when samples were heated at 50°C. Thereafter, samples containing 30 μg of total protein were separated by SDS-PAGE for 60 to 90 min at 200 V using 4–20% gradient gels on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to PVDF membranes at 300 mA constant current for 2 h on ice at 4°C. The homogeneity of protein loading was checked by staining the membrane with Ponceau S. Membranes were blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% non-fat dry milk for 2 h and then incubated overnight at 4°C with commercially available polyclonal primary phosphospecific antibodies to measure the following protein contents with stated dilutions: GAPDH (1:10000; ab9485, Abcam), tubulin (1:1500; T6199, Sigma), PGC-1α (1:4000; 516557, Calbiochem), cyt c (1:500; sc-8385, Santa Cruz biotechnology, Inc.), UCP2 (1:300; ab67241 Abcam), UCP3 (1:3000; ab3477 Abcam) and Total OXPHOS Cocktail (1:1000; ab110413; Abcam). All the antibodies were diluted in TBS-T containing 2.5% non-fat dry milk.
BAT samples were treated as above and prepared for Western-blot analyses for PGC-1α, cyt c, UCP1 (1:4000; ab10983, Abcam), UCP2 and UCP3.

After the primary antibody incubation membranes were washed in TBS-T, incubated with suitable secondary antibody diluted in TBS-T with 2.5% milk for 1 h followed by washing in TBS-T. Proteins were visualized by ECL according to the manufacturer's protocol (SuperSignal West femto maximum sensitivity substrate, Pierce Biotechnology) and quantified using ChemiDoc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories). The UCP3 and pACC membranes described above were incubated in Restore Western blot stripping buffer (Pierce Biotechnology) for 30 min and reprobed with GAPDH or total-ACC antibodies by immunoblot analysis as described above. Results from gastrocnemius muscle were normalized to the corresponding level of GAPDH (PGC-1α, cyt c and UCP3), tubulin (UCP2, total-ACC and pACC) or PonceauS stained actin band (OXPHOS Cocktail). All proteins from BAT samples were normalized to corresponding level of tubulin.

2.2.5 Serum cortisol concentration

Cortisol concentration was measured from frozen (-80 °C) serum samples by a kinetic photometric method with KoneLab (Thermo Scientific).

2.2.6 Ethics statement

This study was approved by the National Animal Experiment Board, Finland (Permit number ESAVI-2010-07989/Ym-23).

2.3 Statistical analyses

All values in figures are expressed as mean ± standard error of the mean (SEM). Statistical analyses for variables were carried out using SPSS for Windows 22 statistical software (version 22, IBM SPSS Statistics) and in Mplus 7. The Shapiro-Wilk test was used to investigate within group normality for a given parameter of interest. Levene's test was conducted to assess the homogeneity of variance assumption. Univariate analysis was done to analyze the line, age, running and treatment effects to the measured parameters with Tukey Post hoc test. When the normality or equality of variance assumptions were not met, statistical comparisons of parameters between LCR and HCR groups were made using Mann-Whitney test. The comparison between before and after intervention parameters within the same group were done using Wilcoxon test. Mixed model analysis controlled for age, running (yes/no), treatment (placebo/glucose), time point (F, 0, 30, 60 and 120 min) and spontaneous activity, was used to determine the effect of line (HCR or LCR) on the measured body temperature levels. Separate analysis for both rat lines of the effect of running, treatment, time point and spontaneous activity on rectal temperature were performed using 4x5 longitudinal covariance structure analyses in Mplus 7 for both glucose tolerance and placebo tests. P-values less than 0.05 were considered statistically significant.

3 Results

3.1 Body temperature

3.1.1 Baseline measurement

HCRs had on average 1.3°C higher body temperature after 2 h of fasting compared to LCRs ($p < 0.001$) (Fig. 2A).

3.2 Intervention measurements

3.2.1 Before and after one-year running intervention measurements

Univariate analysis showed a significant line effect on body temperature both before and after intervention ($p < 0.050$), with HCRs having higher body temperature compared to LCRs (Fig. 2B). When only control groups (HCR vs. LCR) were included in the analyses, the line effect after intervention diminished. Aging did not have a marked impact on body temperature, whereas running had a significant effect after intervention ($p < 0.050$) showing an increase in the body temperature levels in both rat lines ($p < 0.050$, Fig. 2B). Also combined effect of line and running was significant after intervention ($p < 0.050$). Post hoc test showed that after the intervention, HCRs in the runner group had higher body temperature compared to the corresponding controls ($p < 0.010$), whereas there was no difference between the LCR groups (Fig. 2B).

3.2.2 Voluntary running distance, body mass and energy intake

HCRs ran voluntarily more than LCRs, the difference being significant at time points 10, 10.5, 12.5 and 21 months of age ($p < 0.05$) (Fig. 3A). When comparing the body weight, LCR rats in both groups were heavier than HCR rats during the whole follow-up period ($p < 0.05$) (Fig. 3B). There was no statistical difference within the rat lines. LCRs in the control groups had higher food intake compared to corresponding HCRs, the difference being significant at time points 15 and 19.5 months of age ($p < 0.05$). Both runner groups consumed more energy compared to the corresponding control groups (Fig. 3C), but only in HCR line this difference was significant (HCR vs HCR-R, $p < 0.05$).

3.2.3 Glucose tolerance and placebo tests

3.2.3.1 Heat generation

Before the intervention, heat generation was positive when normalized to 0-value during placebo and negative during glucose test, and LCR control group had the largest negative response to glucose injection (Fig. 4A). Univariate analysis showed an effect of treatment on heat generation ($p < 0.001$, Fig. 4A). After the intervention treatment still had a significant effect on heat generation ($p < 0.010$, Fig. 4B) and the effect of line was nearly significant ($p = 0.064$). Responses to both placebo and glucose injections in all groups were negative after the intervention, with both LCR groups having greater negative response to glucose injection compared to HCR groups (Fig. 4B).

3.2.3.2 Spontaneous activity

Total activity during the test protocols are presented in Figures 4C and 4D. Univariate analysis revealed that HCRs had higher total activity during placebo and glucose tolerance tests both before and after the one-year voluntary running intervention compared to LCRs (line effect $p < 0.001$, Fig. 4C and 4D).

3.2.3.3 Blood glucose concentration

Before the intervention the blood glucose AUC value in all groups was negative during placebo test and positive during glucose tolerance test (Fig. 4E). Univariate analysis showed significant treatment effects both before and after the intervention ($p < 0.050$, Fig. 4E and 4F). Post hoc test further revealed that LCRs in the control group had higher blood glucose AUC concentration compared to the corresponding runners both before and after the intervention ($p < 0.050$), whereas there was no statistical difference between the HCR groups.

3.2.4 Serum cortisol concentration

HCRs had higher cortisol concentration compared to LCRs both before and after intervention (line effect, $p < 0.001$, Fig. 5).

3.3 Western-blot analyses

3.3.1 Gastrocnemius muscle

Univariate analysis showed a significant line effect in UCP2 and OXPHOS ($p < 0.050$) with HCRs having higher protein levels compared to LCRs (Fig. 6). HCRs had also higher PGC-1α and cyt c protein levels than LCRs ($p < 0.050$, *reported previously*) (Fig. 6). Aging also increased the level of PGC-1α ($p < 0.050$) and there was a tendency in increase of cyt c level with aging ($p = 0.087$) (*reported previously*). There were no statistical differences in the other studied protein contents.

3.3.2 BAT

Line, running or aging had no significant effect on BAT protein contents (*data not shown*).

3.4 Citrate synthase activity

HCRs had higher citrate synthase activity compared to LCRs (line effect $p < 0.05$, *reported previously*). The activity levels were following: Before intervention: HCR 2939±679, LCR 2767±866 and after intervention: HCR 3267±1081, HCR-R 3554±835, LCR 2863±917 and LCR-R 3545±1328 ($U \bullet \mu g^{-1} \bullet min^{-1}$, mean ± SD). Aging or voluntary running had no significant effect on citrate synthase activity.

3.5 Body mass and relative gastrocnemius muscle and BAT masses

Body and relative masses of gastrocnemius muscle and BAT are listed in Table 1. Before the intervention, HCRs had lower body mass and higher relative gastrocnemius muscle mass compared to LCRs (line effect $p < 0.001$). Aging had a significant effect in both body mass and relative gastrocnemius muscle mass ($p < 0.050$); before the intervention HCRs had higher relative gastrocnemius muscle mass compared to HCRs after the intervention, while LCRs before intervention had higher relative gastrocnemius mass compared to both LCR and LCR-R after intervention. Line, running or age had no significant effect on relative BAT mass.

3.6 Estrous cycle

There were no statistical differences between the groups in the observed stages of estrous cycle during glucose tolerance and placebo tests (Table 2).

3.7 Mixed model and longitudinal covariance structure analysis of body temperature

Mixed model analyses from intervention measurements revealed, that rat line had clear impact on body temperature both before and after intervention ($p < 0.01$, Table 3). Longitudinal covariance analysis showed that before the one-year voluntary running intervention the spontaneous activity level and protocol time point (F, 0, 30, 60 or 120 min) had the largest effect on body temperature of HCRs ($p < 0.05$, Table 3), whereas in LCRs, treatment (placebo/glucose injection) and spontaneous activity had highest impact on body temperature ($p < 0.001$). After the intervention both running and spontaneous activity had a significant contribution to the body temperature levels of HCRs ($p < 0.05$). In LCRs treatment and time point had marked impact on body temperature after the intervention ($p < 0.05$).

4 Discussion

In the present study we examined the association of intrinsic aerobic capacity, aging, voluntary running and blood glucose concentration on thermogenesis in two genetically contrasting rat lines (HCR/LCR) that widely differ for their intrinsic (i.e. non-trained) aerobic capacity (Koch & Britton 2001). Our findings show that at young age, in untrained state, HCRs have higher heat generation i.e. thermogenesis than LCRs. We also found that voluntary running aided HCRs to maintain high thermogenesis during aging. Glucose injection lowered the body temperature of LCRs, whereas no significant effect of blood glucose was found in HCRs.

As hypothesized, our study showed that untrained HCRs had on average 1.3°C higher body temperature than corresponding LCRs (Fig. 2A). There was also an apparent rat line effect when measuring the baseline body temperatures before one-year voluntary running intervention (Fig. 2B). Since normal body temperature range in rats is 35.9-37.5°C (Animal care and use committee, John Hopkins University, Baltimore, Maryland, USA), HCRs seem to have slightly elevated body temperature compared to reference values. Previous findings have shown that female HCRs have higher muscle heat dissipation during activity, explaining their higher total energy expenditure compared to LCRs (Gavini et al. 2014). However, in that study critical factor was activity related thermogenesis, whereas no significant contribution of resting metabolic rate was found when body size and composition were considered (Gavini et al. 2014). As shown in previous study by Novak *et al.* and here, HCRs are spontaneously more active than LCRs (Fig. 4C-D) and activity level also contributed to body temperature level (Table 3), making spontaneous activity a potential cause for the higher thermogenesis of HCRs (Novak et al. 2010). However, the total activity of the rats was very similar during both placebo and glucose tolerance test showing no effect of treatment on activity level (Fig. 4C-D). Yet there was a clear difference in the heat generation between the two protocols (Fig. 3A-B), indicating that the higher heat generation of HCRs is not solely explained by their PA level.

In the present study HCRs had higher UCP2, PGC-1α, cyt c and OXPHOS levels compared to LCRs, showing a greater oxidative phosphorylation capacity (Fig. 6). Interestingly, a previous study has shown that overexpression of PGC-1α in skeletal muscle leads to elevated proton leak i.e. less efficient mitochondrial respiration (St-Pierre et al. 2003). Since HCRs have more OXPHOS proteins and higher UCP2 and PGC-1α levels in skeletal muscle, it may be speculated that the higher proton leakage may also be one reason for HCRs higher thermogenesis. On the other hand, several animal models have demonstrated that rodents with inherited obesity have low body temperatures (Levin, Comai & Sullivan 1981, Trayhurn, Thurlby & James 1977, Dubuc, Wilden & Carlisle 1985). Here we show for the first time, that similarly to inherited obesity, LCRs have lower body temperature than HCRs, and are prone to gain excess weight and develop metabolic disorders (Noland et al. 2007, Koch & Britton 2005, Kivelä et al. 2010).

Contrary to our second hypothesis, aging *per se* had no significant effect on body temperature (Fig. 2B). This is in line with a previous study, where aging did not have an impact on rectal temperatures (3-24 months old), until in later age (36 months) (Horan et al. 1988, McDonald, Stern & Horwitz 1989). However, aging increased the level of PGC-1α and had a tendency to increase cyt c level (Fig. 6), not showing expected aging related decrease of mitochondrial function and content as shown previously (Conley, Jubrias & Esselman 2000, Huang & Hood 2009, Johnson, Robinson & Nair 2013). Despite these unexpected findings from muscle

tissue level, aging did diminish the difference in the heat generation between the rat lines when comparing only the control groups (HCR vs. LCR, Fig. 2B). This was due to decrease in body temperature of HCRs; aging did not have a marked impact on the body temperature of LCRs. For an unknown reason, HCRs seem to lose their ability to keep up high thermogenesis with aging at control conditions (e.g. no running wheel). Our results revealed that aging significantly diminished the relative gastrocnemius muscle mass in HCRs (Table 1), which may partly contribute to the aging related lower heat generation.

As we hypothesized, one-year voluntary running did increase the body temperature in both rat lines showing a significant running effect (Fig 2B). Further analyses showed that running had a significant impact on the body temperature of HCRs (Table 3), which is consistent with their running distances compared to LCRs (Fig. 3A). It has been established in previous studies, that endurance training increases PGC-1α and cyt c levels as well as mitochondrial content and respiratory capacity in skeletal muscle in both humans and rodents (Baar et al. 2002, Booth 1991, Pilegaard, Saltin & Neufer 2003, Holloszy & Coyle 1984). Surprisingly, voluntary running had no significant effect on the mitochondrial proteins in the present study. It seems that in gastrocnemius muscle, there was no pressure to increase the mitochondrial proteins at time point chosen (age of 21 months). Also, as PGC-1α is known to respond to exercise acutely (Baar et al. 2002), so as in our study the running distance decreased gradually over time, also the pressure for change decreased (Fig. 3A). Contrary to the increased body temperature by voluntary running established in our study, in previous studies endurance training has been associated with decreased mRNA expression of the uncoupling proteins in skeletal muscle and reduced thermogenesis in BAT (Nozu et al. 1992, Boss et al. 1998).

It is worth noting that lifespan is generally negatively correlated with body temperature (Rikke & Johnson 2004). Several life-extending manipulations in rodents, such as caloric restriction, have shown to decrease body temperature by 1-5 °C (Rikke & Johnson 2004). Furthermore, a modest prolonged reduction of core body temperature (0.3-0.5°C) increased median life expectancy in mice 12-20 % even without caloric restriction (Conti et al. 2006). Nonetheless, in the HCR/LCR animal model, HCRs have higher body temperature associated with longer lifespan than LCRs (Koch, Britton & Wisloff 2012, Karvinen et al. 2015). The free radical hypothesis of aging includes 'uncoupling to survive' hypothesis, which suggests that correlation between metabolic rate and longevity should be positive (Brand 2000). In a previous study, it was estimated that mitochondrial proton cycling causes up to 20-25% of basal metabolic rate in rats (Rolfe et al. 1999). It was suggested that the function of the energy-dissipating proton leak is not primarily to increase thermogenesis, but to decrease the production of reactive oxygen species. Indeed, proton leak is proposed to be a key factor in aiding to decrease oxidative damage to DNA and to slow down ageing (Brand 2000, Speakman et al. 2004). It seems that HCRs are good candidates supporting the 'uncoupling to survive' hypothesis since their higher OXPHOS, PGC-1α and UCP2 levels combined with higher thermogenesis and longer lifespan compared to LCRs (Koch, Britton & Wisloff 2012, Karvinen et al. 2015).

Previous studies have also shown that core body temperature declines with age both in rodents and in humans (Waalen & Buxbaum 2011, Roth et al. 2002, Sanchez-Alavez, Alboni & Conti 2011), which has raised speculation of possible anti-ageing effects of low body temperature. In our studies, HCRs in the control group had lower body temperature at the age of 21 months than HCR-Rs, and our previous study reported that HCRs in control group had also longer lifespan (Karvinen et al. 2015). According to our results it can be speculated that

voluntary running may interfere with a natural aging-related reduction in body temperature of HCRs, possibly contributing to a decrease in lifespan. However, it remains controversial whether high body temperature and high rate of metabolism are beneficial to health and longevity, and more studies are needed to investigate the role of metabolic rate on aging and longevity.

Our final hypothesis was that glucose injection would increase the body temperature especially in HCRs, increasing heat generation in BAT. Our results revealed, that especially in LCRs, heat generation was lower after glucose tolerance test compared to placebo test (Fig 4A and 4B). Longitudinal covariance analysis further revealed that treatment was a significant contributor to temperature level in LCRs both before and after intervention (Table 3). It may be speculated, that in LCRs glucose injection activates energy storage mechanisms that decrease their metabolic rate shortly after energy supplementation (Almind & Kahn 2004, Ravussin & Gautier 1999, Heikens et al. 2011, Landsberg et al. 2009). However, it should be noticed that there was a difference in the blood glucose AUC level between the LCR groups already before the one year running intervention started (Fig. 4C), which is most likely due to large variation in the response to glucose dose due to small group size. After the intervention rats in the running groups had a lower response to glucose; however, again the post hoc tests revealed that the difference was only significant between the LCR groups (Fig. 3E).

In addition to aging and voluntary running, other potential factors may affect the body temperature in our setup, e.g. handling of the animal and estrous cycle. Handling is known to have a stress effect on rats and it can cause a series of behavioral and physiological responses, such as flight response, freezing response, increase in heart rate, urination and increase in plasma glucocorticoid levels (Rodgers et al. 1997, Blanchard, Griebel & Blanchard 2001). It is known that HCRs have stronger response to stress, and this can be observed also in elevated corticosterone level in blood in HCRs compared to LCRs (Waters et al. 2010). Similarly in our study HCRs had higher cortisol concentration compared to LCRs the (Fig. 5). This stronger response to stress can also be one reason for the higher body temperature of HCRs (Vachon & Moreau 2001). Estrous cycle affects the body temperature in female rats; it increases during proestrus, and drops during estrus (Kent, Hurd & Satinoff 1991, Marrone, Gentry & Wade 1976). In our study the stage of estrous cycle was estimated during the follow-up measurements. Rats were randomly in one of the four estrous phases, and no significant differences in the estrous cycle stages between the rat lines was observed (Table 2). The estrous cycle rhythm does not explain the difference in the body temperatures between the rat lines in our study.

To conclude, HCRs have higher basal body temperature than LCRs at untrained state at young age. Voluntary running aids older HCRs to maintain thermogenesis at similar levels as untrained, younger HCRs. The elevated body temperature itself may partly cause the heightened oxidative metabolism in HCRs, as enzyme-catalyzed reactions are enhanced in higher temperatures (Landsberg et al. 2009). However, voluntary running may interfere with a natural aging-related reduction in body temperature of HCRs, possibly contributing to a decrease in lifespan (Karvinen et al. 2015). On the other hand it is proposed that low body temperature is one reason for the onset of obesity in humans (Landsberg et al. 2009). Yet it remains controversial whether high body temperature and high rate of metabolism are beneficial to health and longevity. However, since there is clear evidence of obese animals having low body temperatures (Levin, Comai & Sullivan 1981, Trayhurn, Thurlby & James 1977, Dubuc, Wilden & Carlisle 1985), it would be worth studying the potential role of intrinsically low body temperature at the onset of obesity in humans.

Conflict interests statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions

HK, MS and SK designed the study. HK led the animal experiment and SK performed the animal experiment and analyzed the data. HM, MS, SK and RT collected the tissue samples. TR and MS built up the spontaneous activity measurement system. TT and SL assisted with the statistical analysis and interpretation of the data. SK, HK and MS drafted the manuscript. SLB and LGK bred and phenotyped the animals. All authors contributed to the revision of the manuscript and approved the final version of the manuscript.

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References

Almind, K. & Kahn, C. R. 2004. Genetic determinants of energy expenditure and insulin resistance in diet-induced obesity in mice. Diabetes 53 (12), 3274-3285.

Baar, K., Wende, A. R., Jones, T. E., Marison, M., Nolte, L. A., Chen, M., Kelly, D. P. & Holloszy, J. O. 2002. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 16 (14), 1879-1886.

Biesiadecki, B. J., Brand, P. H., Koch, L. G. & Britton, S. L. 1999. A gravimetric method for the measurement of total spontaneous activity in rats. Proceedings of the Society for Experimental Biology and Medicine.Society for Experimental Biology and Medicine (New York, N.Y.) 222 (1), 65-69.

Blanchard, D. C., Griebel, G. & Blanchard, R. J. 2001. Mouse defensive behaviors: pharmacological and behavioral assays for anxiety and panic. Neuroscience and biobehavioral reviews 25 (3), 205-218.

Booth, F. W. 1991. Cytochrome c protein synthesis rate in rat skeletal muscle. Journal of applied physiology (Bethesda, Md.: 1985) 71 (4), 1225-1230.

Boss, O., Samec, S., Desplanches, D., Mayet, M. H., Seydoux, J., Muzzin, P. & Giacobino, J. P. 1998. Effect of endurance training on mRNA expression of uncoupling proteins 1, 2, and 3 in the rat. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 12 (3), 335-339.

Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Seydoux, J., Muzzin, P. & Giacobino, J. P. 1997. Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. FEBS letters 408 (1), 39-42.

Brand, M. D. 2000. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Experimental gerontology 35 (6-7), 811-820.

Cannon, B. & Nedergaard, J. 2004. Brown adipose tissue: function and physiological significance. Physiological Reviews 84 (1), 277-359.

Choi, C. S., Fillmore, J. J., Kim, J. K., Liu, Z. X., Kim, S., Collier, E. F., Kulkarni, A., Distefano, A., Hwang, Y. J., Kahn, M., Chen, Y., Yu, C., Moore, I. K., Reznick, R. M., Higashimori, T. & Shulman, G. I. 2007. Overexpression of uncoupling protein 3 in skeletal muscle protects against fat-induced insulin resistance. The Journal of clinical investigation 117 (7), 1995-2003.

Clapham, J. C., Arch, J. R., Chapman, H., Haynes, A., Lister, C., Moore, G. B., Piercy, V., Carter, S. A., Lehner, I., Smith, S. A., Beeley, L. J., Godden, R. J., Herrity, N., Skehel, M., Changani, K. K., Hockings, P. D., Reid, D. G., Squires, S. M., Hatcher, J., Trail, B., Latcham, J., Rastan, S., Harper, A. J., Cadenas, S., Buckingham, J. A., Brand, M. D. & Abuin, A. 2000. Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. Nature 406 (6794), 415-418.

Conley, K. E., Jubrias, S. A. & Esselman, P. C. 2000. Oxidative capacity and ageing in human muscle. The Journal of physiology 526 Pt 1, 203-210.

Conti, B., Sanchez-Alavez, M., Winsky-Sommerer, R., Morale, M. C., Lucero, J., Brownell, S., Fabre, V., Huitron-Resendiz, S., Henriksen, S., Zorrilla, E. P., de Lecea, L. & Bartfai, T. 2006. Transgenic mice with a reduced core body temperature have an increased life span. Science (New York, N.Y.) 314 (5800), 825-828.

Costford, S. R., Chaudhry, S. N., Crawford, S. A., Salkhordeh, M. & Harper, M. E. 2008. Long-term high-fat feeding induces greater fat storage in mice lacking UCP3. American journal of physiology.Endocrinology and metabolism 295 (5), E1018-24.

Dubuc, P. U., Wilden, N. J. & Carlisle, H. J. 1985. Fed and fasting thermoregulation in ob/ob mice. Annals of Nutrition & Metabolism 29 (6), 358-365.

629 Freyssenet, D., Berthon, P. & Denis, C. 1996. Mitochondrial biogenesis in skeletal muscle in
630 response to endurance exercises. Archives of Physiology and Biochemistry 104 (2), 129-
631 141.
632 Gavini, C. K., Mukherjee, S., Shukla, C., Britton, S. L., Koch, L. G., Shi, H. & Novak, C. M.
633 2014. Leanness and heightened nonresting energy expenditure: role of skeletal muscle
634 activity thermogenesis. American journal of physiology.Endocrinology and metabolism
635 306 (6), E635-47.
636 Geiser, F. 1988. Reduction of metabolism during hibernation and daily torpor in mammals
637 and birds: temperature effect or physiological inhibition? Journal of comparative
638 physiology.B, Biochemical, systemic, and environmental physiology 158 (1), 25-37.
639 Gleeson, M. 1998. Temperature regulation during exercise. International Journal of Sports
640 Medicine 19 Suppl 2, S96-9.
641 Heikens, M. J., Gorbach, A. M., Eden, H. S., Savastano, D. M., Chen, K. Y., Skarulis, M. C.
642 & Yanovski, J. A. 2011. Core body temperature in obesity. The American Journal of
643 Clinical Nutrition 93 (5), 963-967.
644 Holloszy, J. O. 2008. Regulation by exercise of skeletal muscle content of mitochondria and
645 GLUT4. Journal of physiology and pharmacology : an official journal of the Polish
646 Physiological Society 59 Suppl 7, 5-18.
647 Holloszy, J. O. & Coyle, E. F. 1984. Adaptations of skeletal muscle to endurance exercise
648 and their metabolic consequences. Journal of applied physiology: respiratory,
649 environmental and exercise physiology 56 (4), 831-838.
650 Horan, M. A., Little, R. A., Rothwell, N. J. & Stock, M. J. 1988. Changes in body
651 composition, brown adipose tissue activity and thermogenic capacity in BN/BiRij rats
652 undergoing senescence. Experimental gerontology 23 (6), 455-461.
653 Huang, J. H. & Hood, D. A. 2009. Age-associated mitochondrial dysfunction in skeletal
654 muscle: Contributing factors and suggestions for long-term interventions. IUBMB life
655 61 (3), 201-214.
656 Huttemann, M., Pecina, P., Rainbolt, M., Sanderson, T. H., Kagan, V. E., Samavati, L., Doan,
657 J. W. & Lee, I. 2011. The multiple functions of cytochrome c and their regulation in life
658 and death decisions of the mammalian cell: From respiration to apoptosis.
659 Mitochondrion 11 (3), 369-381.
660 Johnson, M. L., Robinson, M. M. & Nair, K. S. 2013. Skeletal muscle aging and the
661 mitochondrion. Trends in endocrinology and metabolism: TEM 24 (5), 247-256.
662 Jones, T. E., Baar, K., Ojuka, E., Chen, M. & Holloszy, J. O. 2003. Exercise induces an
663 increase in muscle UCP3 as a component of the increase in mitochondrial biogenesis.
664 American journal of physiology.Endocrinology and metabolism 284 (1), E96-101.
665 Karvinen, S., Waller, K., Silvennoinen, M., Koch, L. G., Britton, S. L., Kaprio, J.,
666 Kainulainen, H. & Kujala, U. M. 2015. Physical activity in adulthood: genes and
667 mortality. Scientific reports 5, 18259.
668 Kent, S., Hurd, M. & Satinoff, E. 1991. Interactions between body temperature and wheel
669 running over the estrous cycle in rats. Physiology & Behavior 49 (6), 1079-1084.
670 Kivelä, R., Silvennoinen, M., Lehti, M., Rinnankoski-Tuikka, R., Purhonen, T., Ketola, T.,
671 Pullinen, K., Vuento, M., Mutanen, N., Sartor, M. A., Reunanen, H., Koch, L. G.,
672 Britton, S. L. & Kainulainen, H. 2010. Gene expression centroids that link with low
673 intrinsic aerobic exercise capacity and complex disease risk. The FASEB journal :
674 official publication of the Federation of American Societies for Experimental Biology 24
675 (11), 4565-4574.
676 Koch, L. G. & Britton, S. L. 2005. Divergent selection for aerobic capacity in rats as a model
677 for complex disease. Integrative and comparative biology 45 (3), 405-415.

678 Koch, L. G. & Britton, S. L. 2001. Artificial selection for intrinsic aerobic endurance running
679 capacity in rats. Physiological genomics 5 (1), 45-52.
680 Koch, L. G., Britton, S. L. & Wisloff, U. 2012. A rat model system to study complex disease
681 risks, fitness, aging, and longevity. Trends in cardiovascular medicine 22 (2), 29-34.
682 Koch, L. G., Kemi, O. J., Qi, N., Leng, S. X., Bijma, P., Gilligan, L. J., Wilkinson, J. E.,
683 Wisloff, H., Hoydal, M. A., Rolim, N., Abadir, P. M., Van Grevenhof, I., Smith, G. L.,
684 Burant, C. F., Ellingsen, O., Britton, S. L. & Wisloff, U. 2011. Intrinsic Aerobic
685 Capacity Sets a Divide for Aging and Longevity. Circulation research .
686 Kontani, Y., Wang, Z., Furuyama, T., Sato, Y., Mori, N. & Yamashita, H. 2002. Effects of
687 aging and denervation on the expression of uncoupling proteins in slow- and fast-twitch
688 muscles of rats. Journal of Biochemistry 132 (2), 309-315.
689 Landsberg, L. 2012. Core temperature: a forgotten variable in energy expenditure and obesity?
690 Obesity reviews : an official journal of the International Association for the Study of
691 Obesity 13 Suppl 2, 97-104.
692 Landsberg, L., Saville, M. E. & Young, J. B. 1984. Sympathoadrenal system and regulation
693 of thermogenesis. The American Journal of Physiology 247 (2 Pt 1), E181-9.
694 Landsberg, L., Young, J. B., Leonard, W. R., Linsenmeier, R. A. & Turek, F. W. 2009. Is
695 obesity associated with lower body temperatures? Core temperature: a forgotten variable
696 in energy balance. Metabolism: clinical and experimental 58 (6), 871-876.
697 Levin, B. E., Comai, K. & Sullivan, A. C. 1981. Metabolic and sympatho-adrenal
698 abnormalities in the obese Zucker rat: effect of chronic phenoxybenzamine treatment.
699 Pharmacology, biochemistry, and behavior 14 (4), 517-525.
700 Levine, J. A., Eberhardt, N. L. & Jensen, M. D. 1999. Role of nonexercise activity
701 thermogenesis in resistance to fat gain in humans. Science (New York, N.Y.) 283 (5399),
702 212-214.
703 Lin, A. C., Liao, C. W., Lin, S. W., Huang, C. Y., Liou, C. J. & Lai, Y. S. 2015. Canine heat
704 shock protein 27 promotes proliferation, migration, and doxorubicin resistance in the
705 canine cell line DTK-F. Veterinary journal (London, England : 1997) .
706 Marrone, B. L., Gentry, R. T. & Wade, G. N. 1976. Gonadal hormones and body temperature
707 in rats: effects of estrous cycles, castration and steroid replacement. Physiology &
708 Behavior 17 (3), 419-425.
709 McDonald, R. B., Stern, J. S. & Horwitz, B. A. 1989. Thermogenic responses of younger and
710 older rats to cold exposure: comparison of two strains. Journal of gerontology 44 (2),
711 B37-42.
712 Noland, R. C., Thyfault, J. P., Henes, S. T., Whitfield, B. R., Woodlief, T. L., Evans, J. R.,
713 Lust, J. A., Britton, S. L., Koch, L. G., Dudek, R. W., Dohm, G. L., Cortright, R. N. &
714 Lust, R. M. 2007. Artificial selection for high-capacity endurance running is protective
715 against high-fat diet-induced insulin resistance. American journal of
716 physiology.Endocrinology and metabolism 293 (1), E31-41.
717 Novak, C. M., Escande, C., Burghardt, P. R., Zhang, M., Barbosa, M. T., Chini, E. N., Britton,
718 S. L., Koch, L. G., Akil, H. & Levine, J. A. 2010. Spontaneous activity, economy of
719 activity, and resistance to diet-induced obesity in rats bred for high intrinsic aerobic
720 capacity. Hormones and behavior 58 (3), 355-367.
721 Novak, C. M. & Levine, J. A. 2007. Central neural and endocrine mechanisms of non-
722 exercise activity thermogenesis and their potential impact on obesity. Journal of
723 neuroendocrinology 19 (12), 923-940.
724 Nozu, T., Kikuchi, K., Ogawa, K. & Kuroshima, A. 1992. Effects of running training on in
725 vitro brown adipose tissue thermogenesis in rats. International journal of biometeorology
726 36 (2), 88-92.

727 Pilegaard, H., Ordway, G. A., Saltin, B. & Neufer, P. D. 2000. Transcriptional regulation of
728 gene expression in human skeletal muscle during recovery from exercise. American
729 journal of physiology.Endocrinology and metabolism 279 (4), E806-14.
730 Pilegaard, H., Saltin, B. & Neufer, P. D. 2003. Exercise induces transient transcriptional
731 activation of the PGC-1alpha gene in human skeletal muscle. The Journal of physiology
732 546 (Pt 3), 851-858.
733 Ravussin, E. & Gautier, J. F. 1999. Metabolic predictors of weight gain. International journal
734 of obesity and related metabolic disorders : journal of the International Association for
735 the Study of Obesity 23 Suppl 1, 37-41.
736 Rikke, B. A. & Johnson, T. E. 2004. Lower body temperature as a potential mechanism of
737 life extension in homeotherms. Experimental gerontology 39 (6), 927-930.
738 Rodgers, R. J., Cao, B. J., Dalvi, A. & Holmes, A. 1997. Animal models of anxiety: an
739 ethological perspective. Brazilian journal of medical and biological research = Revista
740 brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ...[et al.]
741 30 (3), 289-304.
742 Rolfe, D. F., Newman, J. M., Buckingham, J. A., Clark, M. G. & Brand, M. D. 1999.
743 Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle
744 and liver and to SMR. The American Journal of Physiology 276 (3 Pt 1), C692-9.
745 Rosenbaum, M., Hirsch, J., Gallagher, D. A. & Leibel, R. L. 2008. Long-term persistence of
746 adaptive thermogenesis in subjects who have maintained a reduced body weight. The
747 American Journal of Clinical Nutrition 88 (4), 906-912.
748 Roth, G. S., Lane, M. A., Ingram, D. K., Mattison, J. A., Elahi, D., Tobin, J. D., Muller, D. &
749 Metter, E. J. 2002. Biomarkers of caloric restriction may predict longevity in humans.
750 Science (New York, N.Y.) 297 (5582), 811.
751 Rothwell, N. J. & Stock, M. J. 1979. A role for brown adipose tissue in diet-induced
752 thermogenesis. Nature 281 (5726), 31-35.
753 Rousset, S., Alves-Guerra, M. C., Mozo, J., Miroux, B., Cassard-Doulcier, A. M., Bouillaud,
754 F. & Ricquier, D. 2004. The biology of mitochondrial uncoupling proteins. Diabetes 53
755 Suppl 1, S130-5.
756 Russell, A. P., Wadley, G., Hesselink, M. K., Schaart, G., Lo, S., Leger, B., Garnham, A.,
757 Kornips, E., Cameron-Smith, D., Giacobino, J. P., Muzzin, P., Snow, R. & Schrauwen, P.
758 2003. UCP3 protein expression is lower in type I, IIa and IIx muscle fiber types of
759 endurance-trained compared to untrained subjects. Pflugers Archiv : European journal of
760 physiology 445 (5), 563-569.
761 Sanchez-Alavez, M., Alboni, S. & Conti, B. 2011. Sex- and age-specific differences in core
762 body temperature of C57Bl/6 mice. Age (Dordrecht, Netherlands) 33 (1), 89-99.
763 Silvennoinen, M., Rantalainen, T. & Kainulainen, H. 2014. Validation of a method to
764 measure total spontaneous physical activity of sedentary and voluntary running mice.
765 Journal of neuroscience methods 235 (0), 51-58.
766 Son, C., Hosoda, K., Ishihara, K., Bevilacqua, L., Masuzaki, H., Fushiki, T., Harper, M. E. &
767 Nakao, K. 2004. Reduction of diet-induced obesity in transgenic mice overexpressing
768 uncoupling protein 3 in skeletal muscle. Diabetologia 47 (1), 47-54.
769

Speakman, J. R., Talbot, D. A., Selman, C., Snart, S., McLaren, J. S., Redman, P., Krol, E., Jackson, D. M., Johnson, M. S. & Brand, M. D. 2004. Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. Aging cell 3 (3), 87-95.

Stanford, K. I., Middelbeek, R. J., Townsend, K. L., An, D., Nygaard, E. B., Hitchcox, K. M., Markan, K. R., Nakano, K., Hirshman, M. F., Tseng, Y. H. & Goodyear, L. J. 2013. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. The Journal of clinical investigation 123 (1), 215-223.

St-Pierre, J., Lin, J., Krauss, S., Tarr, P. T., Yang, R., Newgard, C. B. & Spiegelman, B. M. 2003. Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells. The Journal of biological chemistry 278 (29), 26597-26603.

Tonkonogi, M., Krook, A., Walsh, B. & Sahlin, K. 2000. Endurance training increases stimulation of uncoupling of skeletal muscle mitochondria in humans by non-esterified fatty acids: an uncoupling-protein-mediated effect? The Biochemical journal 351 Pt 3, 805-810.

Trayhurn, P., Thurlby, P. L. & James, W. P. 1977. Thermogenic defect in pre-obese ob/ob mice. Nature 266 (5597), 60-62.

Vachon, P. & Moreau, J. P. 2001. Serum corticosterone and blood glucose in rats after two jugular vein blood sampling methods: comparison of the stress response. Contemporary topics in laboratory animal science / American Association for Laboratory Animal Science 40 (5), 22-24.

van den Berg, S. A., van Marken Lichtenbelt, W., Willems van Dijk, K. & Schrauwen, P. 2011. Skeletal muscle mitochondrial uncoupling, adaptive thermogenesis and energy expenditure. Current opinion in clinical nutrition and metabolic care 14 (3), 243-249.

Waalen, J. & Buxbaum, J. N. 2011. Is older colder or colder older? The association of age with body temperature in 18,630 individuals. The journals of gerontology.Series A, Biological sciences and medical sciences 66 (5), 487-492.

Waters, R. P., Renner, K. J., Summers, C. H., Watt, M. J., Forster, G. L., Koch, L. G., Britton, S. L. & Swallow, J. G. 2010. Selection for intrinsic endurance modifies endocrine stress responsiveness. Brain research 1357, 53-61.

Wisloff, U., Najjar, S. M., Ellingsen, O., Haram, P. M., Swoap, S., Al-Share, Q., Fernstrom, M., Rezaei, K., Lee, S. J., Koch, L. G. & Britton, S. L. 2005. Cardiovascular risk factors emerge after artificial selection for low aerobic capacity. Science (New York, N.Y.) 307 (5708), 418-420.

Yamashita, H., Yamamoto, M., Ookawara, T., Sato, Y., Ueno, N. & Ohno, H. 1994. Discordance between thermogenic activity and expression of uncoupling protein in brown adipose tissue of old rats. Journal of gerontology 49 (2), B54-9.

Figures

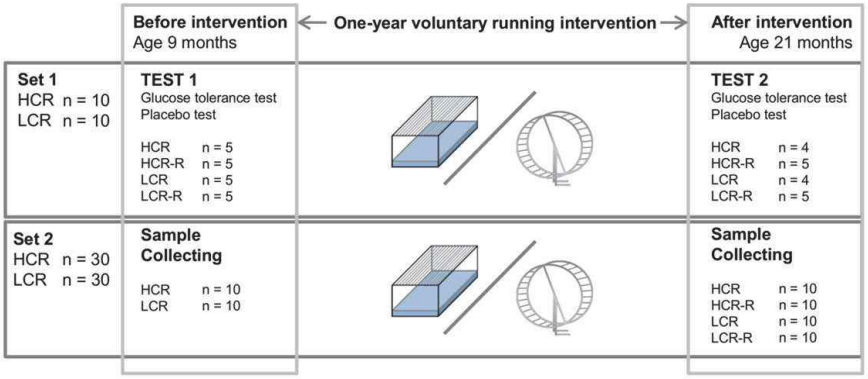

Figure 1

Schematic representation of the study protocol

There were two sets of rats with the same one-year intervention with 4 sub-groups: HCR (control), HCR-R (runner), LCR (control) and LCR-R (runner). For set1 (20 rats) glucose tolerance and placebo tests were performed before the intervention (age 9 months) and after the intervention (age 21 months). From set 2 (60 rats) gastrocnemius muscle and brown fat samples were collected from the same time points.

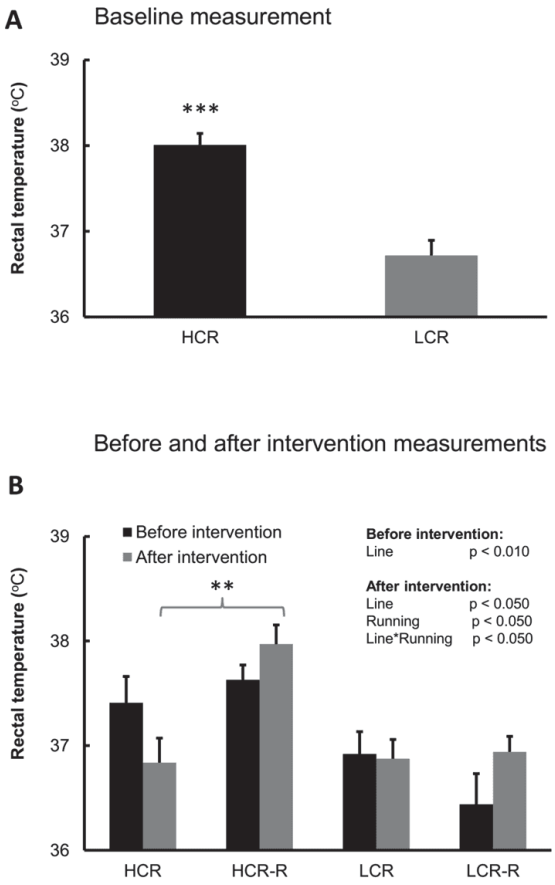

Figure 2

Baseline and before and after intervention measurements of body temperature

Baseline measurement of body temperature (2A). n = 10/group, ***p < 0.001. Before and after intervention measurements of body temperature (2B). n = 4-5/group, **p < 0.010 post hoc test between HCR and HCR-R. Univariate analysis of rat line and running effect before and after one-year intervention. Values are expressed as mean ± SEM.

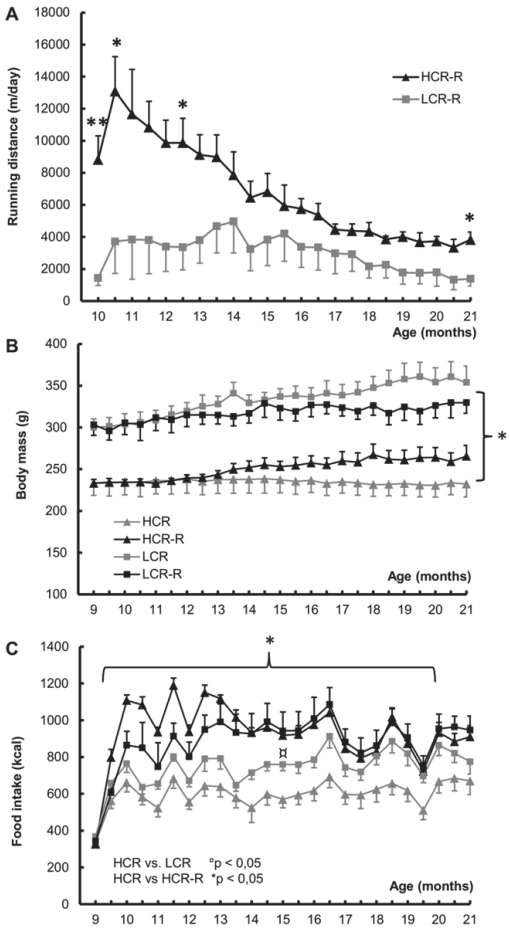

Figure 3

Voluntary running distance, Body mass and Food intake during one year intervention

3A: Voluntary running distance (m/day). HCRs had longer voluntary running distance than LCRs, the difference being significant at time points 10, 10.5, 12.5 and 21 months of age ($p < 0.050$).

3B: Body mass (g). LCR rats in both groups were heavier compared to HCR rat groups during the follow-up period ($p < 0.050$). In LCRs the rats in the runner group were leaner than control ones, whereas the opposite was true for HCRs. There were no statistical differences within the rat lines.

3C: Food intake (kcal). LCRs in the control groups had higher food intake compared to corresponding HCRs, the difference was significant at time points 15 and 19.5 months of age ($p < 0.050$). Runner groups of both rat lines consumed more energy compared to the corresponding control groups, but only in HCR line this difference was significant (HCR vs HCR-R, $p < 0.050$). n = 4-5/group, values are expressed as mean ± SEM.

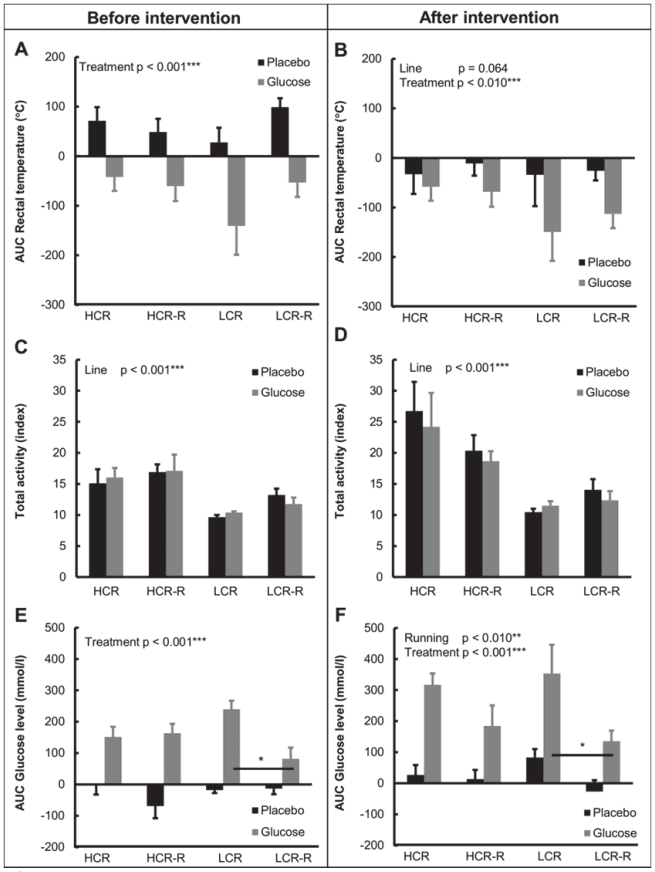

Figure 4

Heat generation (AUC rectal temperature), total activity and blood glucose concentration (AUC) during placebo and glucose tolerance tests

4A and 4B: AUC Rectal temperature. Before the one-year intervention treatment had a significant effect on heat generation ($p < 0.001$), with glucose injection lowering the heat generation. After the intervention treatment was still significant ($p < 0.010$) and line effect was nearly significant ($p = 0.064$).

4C and 4D: Total activity. HCRs had higher total activity compared to LCRs both before and after one-year intervention (line effect $p < 0.001$).

4E and 4F: AUC Glucose concentration.

Treatment had a significant contribution to blood glucose AUC concentration both before and after intervention ($p < 0.001$), with glucose injection increasing blood glucose AUC. Post hoc test revealed that LCRs controls had higher blood glucose AUC compared to the corresponding runners both before and after the intervention ($p < 0.050$).

Univariate analysis of effects of rat line, treatment (glucose/placebo injection) and voluntary running on studied parameters. n = 4-5/group, values are expressed as mean ± SEM.

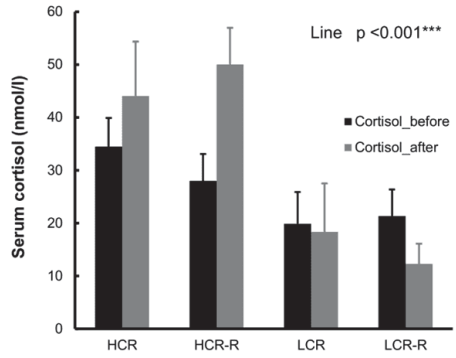

Figure 5

Cortisol

HCRs had higher cortisol concentrations compared to LCRs both before and after intervention (line effect $p < 0.001$).

Serum analyses were done from the fasting blood samples collected before and after intervention. n = 4-5/group, values are expressed as mean ± SEM.

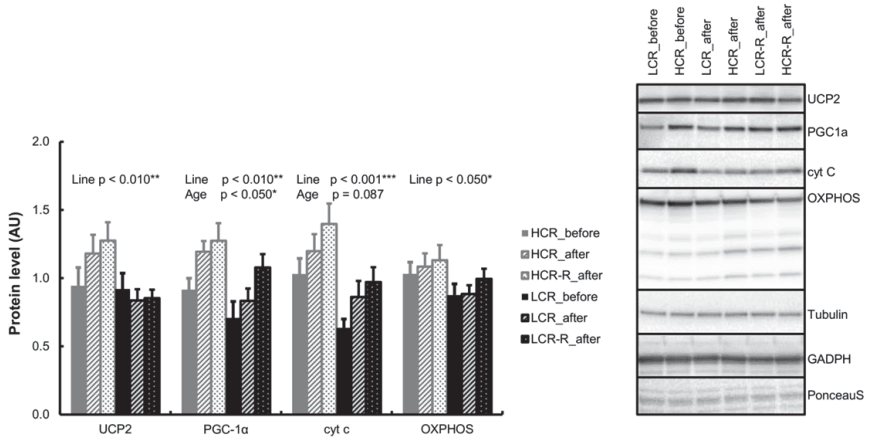

Figure 6

Western blot analyses from gastrocnemius muscle

HCRs had higher UCP2, PGC-1α, cyt c and OXPHOS protein levels (line effect $p < 0.050$). Aging increased the level of PGC-1α ($p < 0.050$) and there was a tendency in increase of cyt c level with aging ($p = 0.087$). n = 9-10/group, values are expressed as mean ± SEM.

Tables

Table 1: Background information for Western blot analyses

	Body mass (g)	Gastrocnemius/Body mass (mg/g)	Brown fat/Body mass (mg/g)
HCR_young	232 ± 30	5,32 ± 0,65	
HCR_old	260 ± 36	4,57 ± 0,58	1,33 ± 0,22
HCR-R old	268 ± 34	4,78 ± 0,49	1,23 ± 0,27
LCR_young	302 ± 26	4,73 ± 0,43	
LCR_old	320 ± 37	4,21 ± 0,45	1,02 ± 0,36
LCR-R_old	345 ± 48	4,00 ± 0,59	1,16 ± 0,26
p	Line < 0.001***	Line < 0.001***	
	Age < 0.050*	Age < 0.001***	

Body and relative tissue masses (tissue mass/body mass) of the rats used for western blot analyses. n = 10/group, values are expressed as mean ± SD.

Table 2: Stage of estrous cycle

N	**HCR**		**HCR-R**		**LCR**		**LCR-R**	
	Placebo	Glucose	Placebo	Glucose	Placebo	Glucose	Placebo	Glucose
Stage of estrous cycle:								
Proestrus	1	1	0	0	1	1	1	2
Estrus	0	0	0	3	1	2	1	2
Metestrus	2	1	2	1	1	1	2	1
Diestrus	1	2	3	1	1	0	1	0

Estimated stage of estrous during the second (age 21 months) glucose tolerance and placebo tests. Numbers are n of animals that are in the presented stage of estrous cycle. There were no statistical differences between the groups.

Table 3: Body temperature during test protocols: effect of measured parameters

		Factor	p
Before intervention		Line	<0.000***
	HCR	Running	0.510
		Treatment	0.067
		Time point	0.036*
		Spontaneous activity	0.037*
	LCR	Running	0.955
		Treatment	< 0.001***
		Time point	0.402
		Spontaneous activity	< 0.001***
After intervention		Line	<0.010**
	HCR	Running	< 0.001***
		Treatment	0.815
		Time point	0.387
		Spontaneous activity	0.014*
	LCR	Running	0.330
		Treatment	0.013*
		Time point	0.010*
		Spontaneous activity	0.370

Mixed model and longitudinal covariance structure analysis of how measured parameters affect absolute body temperature levels during placebo and glucose tolerance tests. Parameters: running (yes/no), treatment (glucose/placebo injection), time point (F, 0, 30, 60 or 120 min) and spontaneous activity before and after voluntary running intervention. n = 4-5 /group.