Rats bred for low aerobic capacity become promptly fatigued and have slow metabolic recovery after stimulated, maximal muscle contractions.

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 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.
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 were 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 2–3 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 $^{31}$P-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 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$_2$O. 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$_2$O. 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**

Injections were performed in a 4.7 T horizontal magnet (Magnex Scientific, Abdingdon, 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
Pulse-acquire technique using 130 spectra from the triceps surae were continuously acquired with a RF coil and muscle metabolic functions were studied with 31P-MRS surface coil to ensure that the position of the muscle was correct. 31P-MR signal followed by pilot images with gradient echo pulse sequence variable. Time-interval showed rapid linear increase in the analyzed area. Difference between Pi and PCr (p = 0.001). 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 and 2093 ± 25 m for HCRs (p < 0.001), representing a 586% difference in running distance.

31P-MRS

PCr. 31P-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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Epoch</th>
<th>LCR</th>
<th>HCR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twitch force</td>
<td>First</td>
<td>2.87±0.25</td>
<td>2.51±0.13</td>
<td>0.206</td>
</tr>
<tr>
<td>(N)</td>
<td>Last</td>
<td>1.01±0.11</td>
<td>1.29±0.10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MRTD</td>
<td>First</td>
<td>184±17</td>
<td>167±10</td>
<td>0.477</td>
</tr>
<tr>
<td>(N·s−1)</td>
<td>Last</td>
<td>75±8</td>
<td>98±9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HRT</td>
<td>First</td>
<td>16.5±0.4</td>
<td>14.1±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(ms)</td>
<td>Last</td>
<td>15.2±0.4</td>
<td>13.5±0.5</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

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. doi:10.1371/journal.pone.0048345.t001
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<0.05) (Fig. 4A). Similarly, LCRs had tendency for higher MRTD (N s−2) 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<0.05). The half relaxation time (Fig. 4C) of the triceps surae was significantly longer in LCRs throughout the stimulation protocol (p<0.05).

**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/
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 protocol, which is vital to 31P-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 T2-weighted MR images, that

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≤0.05). The MRTD values were significantly lower in LCRs compared to HCRs from 2 min 30 s onwards (p≤0.05). LCRs had higher HRT than HCRs throughout the stimulation protocol (p≤0.05). Values are expressed as mean ± SEM.

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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 to HCRs [33]. This tendency was also true in the case
of non-ADP-stimulated and maximally ADP-stimulated respira-
tion. A diminished sensitivity in skeletal muscle may contribute to
the performance differences between LCR and HCR rats, as a conse-
cuence 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 nucleo-
tide 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
muscle [19,32], which might help resist fatigue. An increase in
Ca2+ 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
Ca2+ 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. Interest-
ingly, 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 consistent 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 of importance. Bigger
muscles and perhaps longer muscle fibers in LCRs could explain the
longer relaxation time [42]. Fatigue, due to either Pi, H2PO4-
or Ca2+ 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 become 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 31P-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.