# Mitochondrial biogenesis-associated factors underlie the magnitude of response to aerobic endurance training in rats

Abstract of the PhD thesis

**Orsolya Marton**

Doctoral School of Sport Sciences
University of Physical Education

Supervisor: Dr. Zsolt Radák, professor, DSc

Official reviewers: Dr. Márk Váczi, associate professor, PhD
Dr. Szabó Tamás, private professor, PhD

Head of the Final Examination Committe:
Dr. Gábor Pavlik, professor emeritus, DSc

Members of the Final Examination Committee:
†Dr. Csaba Istvánfi, rector emeritus, CSc
Dr. Ferenc Ihász, professor, PhD

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## 1. Introduction

The level of cardiovascular fitness, as measured by maximal oxygen uptake ($VO_2$max), has become an important biomarker for quality of life, since a high $VO_2$max is strongly associated with decreased incidence of life-style related diseases. It has been suggested that higher $VO_2$max was important to early humans, because it could increase the efficiency of hunting, in which early humans chased the prey animals and as a result greater aerobic capacity could have significant advantage for more and higher quality food. Therefore, it suggested that aerobic exercise, especially running, could be important for the evolution of homo sapiens.

Clinically, exercise capacity, measured by either maximal oxygen uptake ($VO_2$max) or a treadmill running test to exhaustion is a strong predictor of morbidity and survivability. Indeed, studies show that regular aerobic exercise leads to enhanced $VO_2$max and increases the mean lifespan of laboratory animals and humans. However, $VO_2$max is not the only indicator of increased aerobic performance; the adaptive capacity of skeletal muscle to endurance exercise appears to be crucial. Indeed, the quality and quantity of the skeletal muscle mitochondrial network, mitochondrial biogenesis, and the activity of oxidative enzymes are also highly recognized as limiting factors of aerobic endurance capacity.

Studies within twins and families demonstrate that aerobic trainability is a highly heritable trait.

Recently a rat model has been developed via artificial selective breeding, which permits to study the inherited components of low (LRT) and high trainability (HRT). Selection was based on the acquired change in maximal running distance evaluated by a treadmill-running test to exhaustion. In the untrained condition, LRT and HRT rats are similar for exercise capacity. In that study the animals were trained 3 days per week for 8 wk with the running intensity started at 10 m/min up to a maximum speed of 21 m/min. On average, 8 wk of this moderate endurance running training resulted in an 84

± 20 m gain in running capacity. However, the inter-individual training response varied widely from +754 m gain to a −438 m decline (2.7-fold) in running distance.

HRT rats improve on average by 200 meters for distance run whereas those bred as LRT fail to improve and, on average, decline for running capacity by 65 meters for the given moderate intensity training.

A recent study by Lessard et al. showed that skeletal muscle mitochondrial capacity was similar between LRT and HRT in the sedentary state and that LRT produced normal increases in mitochondrial density and function in response to moderate intensity endurance training. Nonetheless, significant differences were noted for exercise-induced angiogenesis and transforming growth factor β signaling in skeletal muscle. Moreover, this study assessed skeletal muscle gene expression and showed that the LRT and HRT differ in their transcriptional responses to the same acute bout of exercise. The differentially expressed genes belonged mostly to biologically functional categories of gene expression, development, cell-cycle regulation, cellular growth, proliferation, and movement.

Based on the above evidence that skeletal muscle remodeling response may be partly responsible for differences in the adaptive exercise response, the purpose of this study was to test the following objectives.

## 2. Objectives of the study

The aim of my doctoral thesis was to find answers to mitochondrial biogenesis-associated factors underlie the magnitude of response to aerobic endurance training in rats.

Trainability is not only important in the elite sports, but also in recreational activities, in which genetics play a key role, so it is important to study whether individuals with different genetics may be related to mitochondrial biogenesis, that affect trainability.

**Hypothesis:**

1. Following the 12-week treadmill running exercise we use, the LRT and HRT groups will be studied to determine a significant difference between the maximum oxygen uptake and running performance of the animals for control and training groups.

2. Changes in workout play a role in redox balance in response to training

3. Adenosine monophosphate-activated protein kinase (AMPK) has a significant effect on skeletal muscle as a result of training.

4. The factors involved in mitochondrial biogenesis explain the different between low responders to training (LRT) and high responders to training (HRT) animals developed by Britton and Koch.

## 3. Methods

### Animals and exercise protocols

Our tests were performed with the 11th generation of selectively breeding male rats (n=27) from genetically heterogeneous rat population (N/NIH strain, n=152). Low response trainers (LRT, n=13) and high response trainers (HRT, n=14) were developed Lauren Gerard Koch and Steven L Britton.

Animals, 12 months of age at the beginning of the study, were divided into control LRT (LRTC, n=6), exercised LRT (LRTE, n=7), control HRT (HRTC, n=6) and exercised HRT (HRTE, n=8) groups.

Animals were provided with a 12 to 12 hour light-dark period at room temperature (22±1 °C), and they were kept in a normal sized cage, two animals per cage, where *ad libitum* was added to food and water. Animals were cared of according to the guiding Principles for the Care and Use of Animals based upon the Helsinki Declaration, 1964.

The first week (5 days for 10 min per day) of 12 weeks long exercise period consisted of teaching the rats how to run on the treadmill, which is a motor-driven treadmill with six separate bands, which was made by *Tektronik Ltd*. on a special order for the Institute of Sport Science. For each introduction session, the treadmill incline was set at 5° slope and speed was gradually increased from 8 to 23 m/min.

At the time of adaptation and later too we cared with the animals that were not willing to run or slipped off the belt as others.

The exercised groups then trained five times a week, 30 min per day for 12 weeks at 70% of their $VO_2$max, and speed was gradually increased from 15 m/min to 25 m/min. $VO_2$max was measured every second week with the use of a special rat ergospirometer system *(Columbus Instruments, Columbus, OH)*. The day of VO2max measured rats did not exercise.

Our aim was to study the effects of endurance training, with the intensity of 70% maximum oxygen uptake based on literature and our data.

Measurement of $VO_2$max followed the protocol used in our previous research. After 10 minutes of calm and 5 minutes of warm up we increased the speed of the treadmill by 5 m/min every 3$^{rd}$ minutes.

This measurement was kept until: 1) the rat's $VO_2$ did not change when speed was increased, 2) the rat could not keep the position on the belt of the treadmill, 3) the respiratory quotient (RQ= $VCO_2/VO_2$)>1. The $VO_2$max measurement was considered complete, the study was stopped and not repeated if at least one of the listed criteria was achieved. Before the test animals' body weight and running distance was measured.

The animals were killed 2 days after the last exercise session to avoid metabolic effects of the final exercise session. The gastrocnemius muscle was quickly excised, weighed, frozen in liquid nitrogen, and stored at – 80 °C.

**Protocols in the laboratory**

Quantitative variations of the proteins for different effects were measured by western blot technique. A previously stored at – 80 °C frozen gastrocnemius muscle tissue was homogenized in lysis buffer containing NP-40 with politron.

The protein concentration of the samples was measured according to the Bradford method with a kit *(Bio-Rad #600-005)*. During western blotting, 10 to 30 µl of the diluted samples of the same protein concentration (5,4 mg/ml) were run and separated on a 8 to 12% (v/v) polyacrylamide gel. After that, proteins were transferred to PVDF membrane by using electrophoresis. After transfer membrane was blocking in 5% milky TBST or 1% BSA at 4 °C. After blocking the membranes were soaked in the primary antibody solutions overnight. After the incubation with the primary antibody the membranes were washed and soaked into the secondary antibody solution. After incubation with the secondary antibody the labelled protein bands were revealed with

the use of Pierce ECL Western Blotting Substrate. For detection, membranes were exposed to x-ray films. Finally the x-ray films were scanned and the protein densities were quantified using ImageJ. On every membrane α-tubulin was used as internal control.

Changes in oxidized protein levels were determined using an Oxyblot Kittet *(Chemicon/Millipore, S7150)* according to the manufacturer's recommendations. Briefly, proteins were derivatized with DNPH for 15 min followed by incubation at room temperature with a neutralization buffer *(Chemicon/Millipore)*. Thus modified proteins were obtained by Western blot technique and the kit's recommendations.

The enzyme citrate synthase (CS) catalyzing the first reaction of the citric acid cycle, which was measured as Shepherd and Garland. Samples of the same protein concentration were loaded in a 96-well transparent microplate in triplet. Following the comparison of the reagents with the sample, the optical density was read at 405 nm in the 0, 1, 2, and 3 minutes with the ELISA reader *(Thermo Labsystems Multiskan EX)*, and the activity was determined in μmol/min/mg protein.

The muscle tissue's $NAD^+$/NADH ratio was determined using Quantification Kit *(Bio Vision, K337-100)* according to the manufacturer's recommendations, which 20 mg tissue was used. According to the guide, first the NADH sample was determined and then the $NAD^+$ in the sample. The $NAD^+$ was estimated from the difference of NADt-NADH after the NADt was detected at 450 nm wavelength with an ELISA reader *(Thermo Labsystems Multiskan EX)* during the 5-hour process every 30 minutes.

The overall ROS generation was determined using $H_2$DCFDA *(Invitrogen-Molecular Probes #D399).* The change in fluorescence intensity was monitored every 5 min for 30 min with excitation and emission wavelengths se tat 485 and 538 nm *(Fluoroskan Ascent FL)*. The fluorescence intensity unit was normalized with the protein content and expressed in relative unit/mg protein.

The messenger RNA levels of AMPKα *(PRKAA1)* was measured using muscle tissue. The total RNA was extracted with NucleoSpin® RNA/Protein (*Macherey-Nagel, Düren, Germany*), and cDNA was determined with *cDNA Syhnthesis Kittel (Bioline, #Biol-65026).* RT-PCR RT-PCR measurement was performed on Rotor-Gene 6000 real-time system *(Corbett Research, Australia).* SYBR Green *(EVA-Green, Biotium, # 31000)* and ImmoMix *(# IMX-110C, Bioline)* were used for the measurement. The thermocycling profile conditions used were: 95 °C for 10 min, 95 °C for 15 seconds, 60 °C for 1 min (40 cycles was uesd). The expression of mRNA of AMPKα was mormalized to β-actin.

**Statistic**

Evaluation of data and significant differences were analyzed in STATISTICA 11.0 after normality. Since a significant part of the variables did not show normal distribution, so for the analysis of all variables there was used non-parametric Kruskall-Wallis ANOVA. After that, post-hoc analysis was performed, based on the non-parametric assay of the 2-sample t-test (Mann-Whitney test). For some data we used a non-parametric two-sample t-test. Significance levels are reported for $p<0,05$ és $p<0,01$.

## 4. Results

The weight of the animals was measured weekly during the exercise period. The initial body mass of the LRT and the HRT group was similar. From the third measurement time, the body mass of the "training resistant" animals and the "trainable" animals body weight decreased continuously. The body weight of LRTE animals was significantly lower than the baseline values at the end of the training period ($p < 0.05$) compared to the LRTC group (422.14 ± 15.19 vs. 474.00 ± 14.10 g), and similar changes were observed in HRTE and HRTC groups (410.63 ± 9.52 versus 471.00 ± 12.88 g).

Before exercise training, maximal oxygen consumption ($VO_2max$) was similar between all four experimental groups - LRTC, LRTE, HRTC and HRTE – and was on avarage ~ 65 ± 7.5 ml / kg / min. Aerobic exercise training significantly increased VO2max in both LRTE and HRTE groups ($p<0,05$) compared to control groups. However, the increase was more significantly from the 3rd measurement time between exercised and control groups, moreover the increase was more enhanced in HRTE animals ($p < 0.01$) than in LRTE animals during the last week of the training protocol ($p<0.05$), which was analyzed by non-parametric two-sample t-test.

The running distance measured during the $VO_2max$ test was similar between the four experimental groups before training and increased significantly in the trained groups. However, there was a significant differential for the change in running distance between LRTE and HRTE groups. HRTE groups ran more than 20 5 longer than LRTE animals during the final treadmill running test.

The amount of mitochondria of skeletal muscle was appraised by the COX-4 content, increased significantly between the LRTC vs. LRTE and HRTC vs. HRTE groups.

The activity of the citrate synthase (CS) enzyme, as mentioned above, was used to measure the oxidative capacity of mitochondria. Similarly to CS activity, COX-4 was also significantly different between the LRT and the HRT control and trainer group. There are so many differences here that there is a counter-direction to the "training-

resistant" control and training group, since a significant decrease has occurred. In addition, a significant difference was found between the LRTE and HRTE groups, the trainer's "trainable" animals reacted more to the training than the "training resistant" exercised animals. Unlike COX-4 compared to CS only HRTE animals were significantly different in compared to HRTC vs. HRTE and LRTE vs. HRTE ($p < 0.05$). Interestingly, CS activity was significantly lower in exercised LRT than in control LRT animals.

Most of the reactive oxygen species are by-products of the mitochondrial respiration process, which level was measured using the $H_2$DCFDA method. Significantly lower levels were measured in the HRT control group compared to control LRT (14% was the difference between HRTC and LRTC groups). Exercise training tended to increase levels of ROs for both LRT and HRT.

We have found an opposite relationship to the ROS level in the $NAD^+$/NADH ratio - which is used to determine the redox balance - because only the HRTE and LRTE groups were able to detect significant differences. In the HRTC group, the $NAD^+$/NADH ratio was higher than in the LRTC group.

Oxidative stress on proteins in cells and tissues that may be induced by exercise may be estimated by the amount of reactive carbonyl derivatives. Based on our data, we found a significant difference between HRTE and HRTC, while in the LRTE group there was no change compared to the "training resistant" control group.

Among the enzymes involved in the degradation of damaged proteins, the proteasome is one of the major proteins disrupting enzymes. As a result of exercise, the amount of R2 subunit we measured by the proteasome (PSMA6) was significantly increased both in LRT and HRTgroups by exercise.

While the proteasome is responsible for the breakdown of damaged proteins in the cytosol, the Lon protease performs the same task in the mitochondria. As a result of

exercise, only significant difference was observed in the HRT group, no change in the LRT group. Furthermore, there is a significant difference between LRTE and HRTE.

HSP78 is a mitochondrial chaperone that defines defective proteins in mitochondria for degrading enzymes. There was no change in HSP78 level within the four experimental groups.

AMPKα activity is an important indicator of skeletal muscle adaptation, which is estimated by the ratio of pAMPKα/AMPKα. Significant increases was observed in the HRTC group compared to the LRTC and to the HRTE group. The amount of AMPKα protein in the skeletal muscle was also measured by the AMPKα mRNA level, but no differences were found between the groups.

SIRT1 is a sensitive marker of metabolic stress. No differences were found between control groups (LRTC vs. HRTC), but by training, both exercised groups (LRTE and HRTE) were significant differences.

One of the most important element of mitochondrial biogenesis is PGC1-α, known to be elavated in response to exercise training. However, growth was only measured in the HRTE group, while the level of PGC1-α did not change between LRTC and LRTE.

PGC1-α regulates the TFAM and NRF-1 proteins involved in mitochondrial biogenesis. Similar to the pattern for PGC1-α, NRF1 increased only in HRTE group, while no differences were found between LRTC and LRTE.

TFAM levels were significantly lower in the LRTE groups compared to LRTC group. In case of HRT animals, exercise training increased the levels of TFAM.

The quality control of mitochondria is partly regulated by fission and fusion. Fis1 is partly responsible the mitochondrial fission process, which levels was significantly lower in HRTC animals compared to LRTC As a result of exercise, both groups (LRT

and HRT) showed an increasing tendency compared to the control group, although no significant differences were detected.

Mfn1 is responsible for the mitochondrial fusion and changed opposite to Fis1. Mfn1 contents were not different in control conditions but decreasd significantly with exercise training in both experimental groups.

## 5. Discussion

Trainability is a critical issue in high level sport, but it could be also important for the health benefits of daily physical activity. A complex mixture of gene-environment interactions contributes to the large range in training-induced adaptations and creates a considerable challenge for identifying the mechanistic connection between exercise capacity and human health. Here, we used a contrasting rat model system, which was developed by artificial selective breeding to segregate animals into lines of low and high training response, thus allowing us to study trainability in an unbiased mechanistic way.

Before training, there is no significant differences for $VO_2$max or running distance in rats selected for low versus high response to training, which suggests that trainability is not strongly dependent upon baseline $VO_2$max. The differences in response to aerobic exercise training between the LRT and HRT were greater for running distance compared to $VO_2$max, indicating the limited trainability of VO2max reported previously in human studies. Further, it supports data from humans demonstrating that training adaptations for improvement in aerobic performance and aerobic capacity can be uncoupled.

The mitochondrial network is crucial for coping with the metabolic challenge provided by physical exercise. The pioneering study of Holloszy showed that exercise training increases the activity of a number of mitochondrial enzymes and the content of mitochondria.

As can be seen from the results, the initial ROS level was significantly lower in HRT animals than in LRT animals. ROS can be produced not only from muscle cells, but also out of muscle cell sources. Excessive stress can result in muscle damage that may lead to the activation of neutrophils and macrophages through interferon-γ, interleukin-1 and tumor necrosis factors. These immune cells largely produce ROS, which are central elements of the neutrophil defense line. In our study, the differences in the ROS initial level of the animals of the two control groups were found, which were not exposed to any load beyond the two-week running $VO_2$max measurement. In addition, the animals were decapitated 2 days after the last exercise was completed to avoid acute metabolic

effects. However, it is possible for this reason that a higher ROS level was detected in the control group of LRT animals. Since we did not measure interferon-γ, interleukin and tumor necrosis factors, we can not support this possibility so we can not give a precise answer to the difference between the two control groups. The ROS level was measured using $H_2$DCFDA, which did not show any difference between HRTE and LRTE.

Indeed, ROS are involved in a wide range of signaling processes, and redox homeostasis is closely linked to cellular metabolism. In this study, we found an inverse relationship between ROS levels and $NAD^+$/NADH ratio in control versus trained rats selectively bred for high response to training (i.e., HRTC and HRTE groups), suggesting controlled redox homeostasis. This expected relationship was missing in LRT groups. On the other hand, significant differences were not found for COX-4 levels, suggesting that the possible differences in redox balance did not significantly affect the rate of mitochondrial biogenesis.

For COX-4, significant growth was only measured between LRTC and LRTE, as well as between HRTC and HRTE, which is the result of collision with Short and colleagues, who reported a similar change in the 16-week aerobic exercise. Citrate synthase and cytochrome c oxidase activity increased, but COX-4 mRNA levels, as well as levels of PGC-1α, NRF-1 and TFAM is not.

We have observed a significant increase in the levels of carbonylated proteins in HRTE rats compared to HRTC animals, while in the case of LRT animals we did not found. Protein carbonylation is a type of protein oxidation promoted by ROS, when ROS level increases, the level of carbonylated protein increases, too. Significant degree of protein carbonylation was used as a marker of oxidative damage of proteins; however, moderate degree of carbonylation could be associated with the degree of protein turnover. Thus, protein carbonylation indicates that damaged proteins are degraded to repair because carbonylation is an irreversible/irreparable modification. The degradation of proteins was evaluated by the contents of R2 subunit of proteasome and Lon protease; indeed,

regular exercise can elevate the levels of these housekeeping proteins. This is an important process because oxidative modification of proteins results in loss of function.

The lack of differences between LRT and HRT groups on proteasome induction could indicate that the housekeeping of aberrant proteins in the cytosol maybe independent from trainability. However, this was not the case for mitochondrial degradation of oxidized proteins, since LonP was induced only in HRTE groups compared to LRTC and HRTC groups. The Lon levels tend to be lower in LRTC than in HRTC rats ($p=0.22$), which might explain the differences in carbonylated proteins in these groups. However, based on our earlier finding that aging downregulates Lon in skeletal muscle, and exercise can attenuate this effect and, in turn, increase endurance performance. Therefore, it cannot be ruled out that the housekeeping role of Lon protease influences trainability

It is worth knowing from the AMPK that it is activated according to the intensity of the exercise, which has been observed at ≈ 60% of the maximum aerobic capacity while others have found a prolonged effect of AMPK activity at low intensity. The activity of AMPK is an important signaling molecule for endurance exercise activity, and stimulating AMPK via AICAR administration has been shown to enhance aerobic performance. Interestingly enough, we found in nontrained control conditions that the HRT rats have significantly higher activity of AMPK than LRT rats suggesting a greater potential for metabolic responsiveness. Therefore, it cannot be ruled out that high inherent levels of AMPKα activity provide a favorable metabolic base for high trainability to aerobic exercise. We did not find significant differences at the mRNA levels of AMPKα among the groups, but the variation between mRNA and protein levels is not very surprising, due to the degradation of mRNA, impaired transcription, regulation by miRNA, or altered degradation of proteins. A study by Lessard and coworkers reported that AMPK was normally activated by an acute bout of exercise in both LRT and HRT and unlikely to be related to the differential for exercise-induced adaptation response. The decreased AMPK activities in LRT along with a reduction in citrate synthase in response to training suggest that mitochondria-associated factors could be important for trainability. Indeed, a reduction of the beta subunits of AMPK in

skeletal muscle can result in impaired exercise tolerance without significant alteration of mitochondrial contents or sugar metabolism.

We also found that exercise training induced SIRT1 in both low and high response trainers, suggesting that the differential in trainability between LRT and HRT is independent from SIRT1 activity. Of interest is the finding that the PGC1-α content increased only in HRTE group. Currently, there are conflicting data on the involvement of SIRT1 on the activation of PGC1-α. Some reports including a recent paper from Holloszy's group suggest that deacetylation of PGC1-α inhibits the activity of SIRT1 and mitochondrial biogenesis, while other papers indicate that SIRT1-mediated deacetylation activates PGC1-α.

The aim of the present investigation was to study the idea that mitochondrial biogenesis may contribute to the differential in response to training. PGC1-α, NRF1, and TFAM regulate mitochondrial biogenesis. As described above, PGC1-α was increased in the high responsive HRT group but not in the low responsive LRT group in response to exercise training. TFAM levels decreased with exercise training in LRT animals and increased in the HRT group. TFAM has been linked to higher aerobic endurance which we also measured to be greater in HRTE groups. The adverse training response of TFAM in LRT compared to HRT therefore puts this protein on the list of potentially limiting factors for exercise resistance. Moreover, the change of Lon content paralleled TFAM levels. Lon protease is involved in the stability, replication, transcription, and translation of proteins and targets TFAM, steroidogenic acute regulatory protein (StAR), and aconitase for degradation. In addition, NRF1 pattern was also similar to the pattern for PGC1-α; NRF1 was induced with training in HRT rats but not in LRT rats. Hence, downstream response elements of PGC1-α increased only in those animals that showed sensitivity to aerobic training.

Based on our results, the following answers can be given to hypotheses:

1. After the 12-week exercise training we used, the LRT and HRT groups will be studied with a significant difference between the maximum oxygen uptake and running distance of the animals in the control and training groups. **Compared to**

**control groups, both LRTE and HRTE groups showed significant increases in maximum oxygen uptake and running distance. Therefore, a significant difference was not observed in the maximum oxygen uptake, so this part did NOT become true while the run distance was TRUE our hypothesis, as there was a significant difference between the two training groups.**

2. Changes in workout play a role in redox equilibrium in responses to training**. It is NOT true because we did not find a clear oxidative markers that would explain different traits. Although the level of ROS was significant in the control group of LRT and HRT.**

3. Adenosine monophosphate-activated protein kinase (AMPK) has a significant effect on skeletal muscle training**. TRUE, because between HRTC and LRTC animals was a significantly higher the AMPK activity level, which is a greater potential for sensing metabolic changes. In addition, AMPK with decreasing tendency and significantly reduced citrate synthase activity in LRTE animals also amplifies this observation**

4. The factors involved in mitochondrial biogenesis explain the different trainability of LRT and HRT animals. **TRUE, because the studied PGC-1α, NRF-1 and TFAM proteins, which are involved in the mitochondrial biogenesis, significantly increased HRT activity while in the LRT group not.**

Based on these, we can say that trainability is importante for everyday physical activity and elite athletes, too. On the basis of trainability, selectively breeding rats low response trainers (LRT) and high response trainers (HRT) were conducted to assess the effect of endurance training on mitochondrial biogenesis. The animals participated in a 30 minute treadmill exercise for 12 weeks, 5 days a week, where speed was set to 70% of $VO_2$max.

As expected, a significant difference was measured at the running distance between LRT and HRT animals. However, it can be stated that $VO_2$max, COX-4, redox

homeostasis markers (reactive oxygen radicals (ROS)), silent-informator-regulator 1 (SIRT1), $NAD^+$/NADH ratio, proteasome (R2 subunit) and mitochondrial network maintenance mitochondrial fission protein (Fis1) and mitochondrial fusion protein (Mfn1) markers do not clearly affect the different in trainability in LRT and HRT. On the other hand, we can conclude that the difference in the activity of AMP-acitvated protein kinase alpha (AMPKα) and the endurance-induced change is citrate synthetase, carbonylated protein, peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1-α) nuclear respiratory factor (NRF1), mitochondrial transcription factor A (TFAM) and Lon protease may be a limiting factor in the rat population selected on the trainability.

Based on our results, we can conclude that mitochondrial biogenesis-related factors adapt in a different way to endurance training in low response trainers and high response trainers rats.

## 6. Bibliography of own publications

### 6.1 In connection with the thesis


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### 6.2. Not in connection with the thesis


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