# Oxidative Damage in Heart, Brain, and Skeletal Muscle in the Context of Aging, Sex, and Inherited Exercise Capacity

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# 1 List of abbreviations:

| | |
|---|---|
| ANOVA | Analysis of Variance |
| BSA | Bovine Serum Albumin |
| Cu- Zn- SOD | Cupper- Zink- Superoxide Dismutase |
| DNA | Deoxyribonucleic acid |
| DNPH | 2,4-Diphenylhydrazone |
| DTT | Dithiothreitol |
| EDTA | Ethylendiaminetetraacetic acid |
| GPx | Glutathione peroxidase |
| GSH | Glutathione |
| GSSG | oxidized glutathione |
| $H^+$ | Hydrogen ion |
| $H_2O_2$ | Hydrogen peroxide |
| HCL | Hydrochloric acid (Hydrogen chloride) |
| HCR | High Capacity Runners |
| $K_2HPO_4$ | Dipotassium hydrogen phosphate trihydrate |
| KCN | Potassium cyanide |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| LCR | Low Capacity Runner |
| Mn-SOD | Manganese superoxide dismutase |
| MTP | Microtiter plate |
| NADP | Oxidized nicotinamide adenine dinucleotide phosphate |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |
| NBT | Nitroblue Tetrazolium |
| NaF | Sodium flouride |
| NaCl | Sodium chloride |
| NF- B | Nuclear factor of activated B-cell |
| $O^{2-}$ | Superoxide anion |
| $OH^-$ | Hydroxide ion |

| | |
|---|---|
| ROS | Reactive Oxygen Species |
| SDS | Sodium Dodecyle Sulfate |
| SOD | Superoxide dismutase |
| TRIS | Trisaminomethane |

**Units**

| | | |
|---|---|---|
| Time | millisecond | (ms) |
| | second | (s) |
| | minute | (min) |
| Length | millimeters | (mm) |
| | meters | (m) |
| Mass | micrograms | (µg) |
| | milligrams | (mg) |
| | grams | (g) |
| | killograms | (kg) |
| Volume | microliters | (µl) |
| | milliliters | (ml) |
| | liters | (l) |
| Quantity | micromoles | (µmol) |
| | millimoles | (mmol) |
| | Moles | (mol) |
| Current | Milliamperes | (mA) |
| Voltage | Volt | (V) |
| Activity | Unit | (U) |

## 2 Abstract:

**Background** As life expectancy rises, the number of patients with age-related conditions such as cardiovascular diseases, neurodegenerative diseases, and sarcopenia grows. One of the most accepted theories to explain the mechanism of aging is the accumulation of oxidative damage over time. Oxidative stress, defined as the imbalance between reactive oxygen species production and the antioxidant defenses, can result in macromolecular damage of lipids, proteins, and DNA. Over time, this eventually leads to progressive loss of tissue and organ function, a main feature of aging. However, the factors affecting this complex process are still poorly understood. It has been suggested that sex and inherited exercise capacity are two defining factors in the body's response to aging and its corresponding pathologies. Women live longer and develop many diseases with a 10-year delay compared to men. Similarly, high inherited exercise capacity has been associated with a higher life expectancy. Since the influence of the intrinsic (inherited) exercise capacity is hard to investigate in humans separately from the extrinsic form, a specifically bred rat model for high (HCR) and low (LCR) intrinsic aerobic running capacity was developed. In fact, HCRs showed to live almost one third longer than LCRs.

**Objective** We aimed to investigate the antioxidant defense system and oxidative damage markers in the context of aging, sex, low and high inherited exercise capacity.

**Methods** Heart, brain, and gastrocnemius muscle tissues of each female and male rats were tested in 4 different groups: young (4 months old), old (24 months old), HCRs & LCRs. Antioxidant capacity was evaluated through photometric measurements of the 3 most important antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). On the other hand, oxidative damage markers related to lipid peroxidation (4-hydroxynonenal: 4-HNE) and protein carbonylation (PCO) were quantified by western blots and spectrophotometric measurements, respectively.

**Results** In hearts, females presented a similar basal CAT activity between young HCR and LCR rats. A significant increase in enzyme activity with age was observed in both phenotypes. However, this increase was stronger in LCRs, leading to higher enzyme activity in LCRs compared to HCRs within the old females. On the other hand, males exhibited a similar CAT activity among all groups. The other two antioxidant enzymes

(SOD & GPx) did not show any significant differences. In terms of oxidative damage markers, both sexes presented similar PCO and 4-HNE basal levels in young HCR and LCR rats. With age, PCO level was significantly elevated in both phenotypes. However, 4-HNE level increased with age in HCRs only in both sexes.

In brains, both phenotypes showed similar patterns. Females exhibited a significant elevation of CAT activity with age, whereas SOD activity remained unchanged. On the other hand, males showed no significant differences in CAT activity, whereas SOD activity was remarkably reduced with age in both phenotypes. GPx activity showed no significant differences. Both sexes presented a strong elevation of PCO and 4-HNE with age in both HCRs and LCRs.

In gastrocnemius muscles, both sexes showed similar patterns. CAT activity, basally similar, presented a remarkable age-related elevation in HCRs only. LCRs maintained a relatively unchanged enzyme activity with age. This led to a significantly higher CAT activity in old HCRs compared to old LCRs. SOD and GPx activities showed no significant changes. PCO level was strongly elevated with age in both HCRs and LCRs. 4-HNE level did not present any relevant variations with age.

**Conclusion** Genetic predisposition to high exercise capacity was not associated with decreased oxidative damage in heart and brain. Higher protection of HCRs might be assumed in the skeletal muscle due to a higher antioxidant capacity. Furthermore, both sexes presented similar patterns of oxidative damage during aging.

## 3 Zusammenfassung:

Mit steigender Lebenserwartung nimmt die Zahl der Patienten mit altersbedingten Erkrankungen zu. Eine der am meisten akzeptierten Theorien zur Erklärung des Mechanismus des Alterns ist die Anhäufung oxidativer Schäden im Laufe der Zeit. Oxidativer Stress, definiert als das Ungleichgewicht zwischen der Produktion reaktiver Sauerstoffspezies (ROS) und der antioxidativen Abwehr, kann zu einer makromolekularen Schädigung von Lipiden, Proteinen und DNA führen. Im Laufe der Zeit führt dies schließlich zu einem fortschreitenden Verlust der Gewebe- und Organfunktion, ein Hauptmechanismus des Alterns. Die Faktoren, die diesen komplexen Prozess beeinflussen, sind jedoch noch wenig bekannt. Es wurde vermutet, dass Geschlecht und vererbte körperliche Leistungsfähigkeit eine wichtige Rolle im Alterungsprozess und bei den entsprechenden Pathologien spielen. Frauen leben länger und entwickeln viele Krankheiten mit einer Verzögerung von zehn Jahren im Vergleich zu Männern. In ähnlicher Weise wurde eine hohe vererbte Ausdauerkapazität mit einer höheren Lebenserwartung in Verbindung gebracht. Da der Einfluss der intrinsischen (genetisch bedingten) Ausdauerkapazität beim Menschen unabhängig von der extrinsischen (trainierten) Form schwer zu untersuchen ist, wurde ein speziell gezüchtetes Rattenmodell für eine hohe (HCR) und eine niedrige (LCR) intrinsische aerobe Laufkapazität entwickelt. Tatsächlich lebten HCRs fast ein Drittel länger als LCRs.

Ziel dieser Arbeit war es, das antioxidative Defenssystem und die Marker für oxidative Schäden im Zusammenhang mit Alterung, Geschlecht, geringer und hoher vererbter körperliche Leistungsfähigkeit zu untersuchen.

Herz-, Gehirn- und Gastrocnemius-Muskelgewebe aller weiblichen und männlichen Ratte wurden in 4 verschiedenen Gruppen getestet: jung (4 Monate alt), alt (24 Monate alt), HCRs und LCRs. Die Antioxidant-kapazität wurde durch photometrische Messungen der 3 wichtigsten antioxidativen Enzymaktivitäten untersucht: Superoxiddismutase (SOD), Katalase (CAT) und Glutathionperoxidase (GPx). Andererseits wurden oxidative Schadensmarker, die mit der Lipidperoxidation (4-Hydroxynonenal: 4-HNE) und der Proteincarbonylierung (PCO) zusammenhängen, durch Western Blots bzw. spektrophotometrische Messungen quantifiziert.

In den Herzen zeigten Weibchen eine ähnliche basale CAT-Aktivität zwischen jungen HCR- und LCR-Ratten. Bei beiden Phänotypen wurde ein signifikanter Anstieg der Enzymaktivität mit dem Alter beobachtet. Dieser Anstieg war jedoch bei LCRs stärker, was zu einer höheren Enzymaktivität bei LCRs im Vergleich zu HCRs bei den alten Weibchen führte. Andererseits zeigten Männchen in allen Gruppen eine ähnliche CAT-Aktivität. Die beiden anderen antioxidativen Enzyme (SOD & GPx) ergeben keine signifikanten Unterschiede. In Bezug auf oxidative Schadensmarker zeigten beide Geschlechter ähnliche PCO- und 4-HNE-Basalwerte zwischen jungen HCR- und LCR-Ratten. Mit zunehmendem Alter war der PCO-Spiegel in beiden Phänotypen signifikant erhöht. Der 4-HNE-Spiegel zeigte jedoch nur einen altersbedingten Anstieg der HCRs bei beiden Geschlechtern.

Im Gehirn war dennoch bei beiden Phänotypen ähnliche Muster zu beaobacten. Weibchen zeigten eine signifikante Erhöhung der CAT-Aktivität mit dem Alter, während die SOD-Aktivität unverändert blieb. Dagegen zeigten männliche Ratten keine signifikanten Unterschiede in der CAT-Aktivität, während die SOD-Aktivität mit dem Alter bemerkenswert reduziert war bei beiden Phänotypen. Die GPx-Aktivität blieb unverändert. Beide Geschlechter zeigten mit zunehmendem Alter einen starken Anstieg von PCO und 4-HNE sowohl bei HCRs als auch bei LCRs.

In den Gastrocnemius-Muskeln wiesen beide Geschlechter ähnliche Muster auf. Die CAT-Aktivität mit ähnlichen Basalwerten zwichen beiden Phänotypen zeigte nur eine relevante altersbedingte Zunahme der HCR. Die LCRs behielten mit steigendem Alter eine relativ unveränderte Enzymaktivität. Dies führte zu einer signifikant höheren CAT-Aktivität bei alten HCRs im Vergleich zu alten LCRs. SOD- und GPx-Aktivitäten zeigten keine signifikanten Veränderungen. Der PCO-Spiegel war bei beiden HCRs und LCRs mit dem Alter stark erhöht. Der 4-HNE-Spiegel zeigte jedoch keine relevanten Unterschiede.

Die Ergebnisse lassen darauf schließen, dass eine genetische Prädisposition für hohe aerobe Leistungsfähigkeit nicht im Zusammenhang mit einer verminderten oxidativen Schädigung von Herz und Gehirn steht. Aufgrund einer höheren antioxidativen Kapazität kann ein höherer Schutz der HCRs im Skelettmuskel angenommen werden. Darüber hinaus zeigten beide Geschlechter ähnliche Muster oxidativer Schäden während des Alterns.

# 4 Introduction:

According to the latest population estimates, the number of old people around the globe will almost double by 2050 from 2019 numbers (United Nations, 2019). The rapid growth in life expectancy and the aging population is accompanied by a parallel rise of age-related diseases. This imposes a major burden on global health, which requires developing a deeper understanding of the aging process and its related conditions. Recent research is considering oxidative stress to be highly implicated in most of the age-related diseases (Liguori et al., 2018).

## 4.1 Age-related pathologies in heart, brain and skeletal muscles

Cardiovascular diseases are considered the leading cause of death in the aging population (Jaul & Barron, 2017). Due to the high aerobic metabolism of the heart, mitochondrial dysfuction and oxidative stress have been pointed out as relevant factors contributing to heart aging and in the development of cardiac diseases (Peoples et al., 2019). During aging, cellular processes related to mitochondrial function including oxidative stress regulation are negatively altered, which causes cardiac dysfunction (Martín-Fernández & Gredilla, 2018). In fact, the accumulation of oxidative stress over time is considered a major stimulant of pathological remodeling in the aging heart (Rababa'h et al., 2018). This repetitive pattern contributes to several cardiovascular conditions during aging such as atherosclerosis, cardiac hypertrophy and heart failure (Garcia et al., 2017).

Neurodegenerative diseases are another prevalent group in the age-related diseases. The accumulation of oxidative damage in the aging brain is involved the development of many neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Carvalho & Moreira, 2018; Hardy & Higgins, 1992). "The iron hypothesis" has gained a special attention as an underlying mechanism in the literature. It is based on the accumulation of iron with aging that through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$) produces highly reactive hydroxyl radicals, causing damage to DNA, lipid and protein (Egana et al., 2003; Ke & Ming Qian, 2003; Zecca et al., 2004). Furthermore, oxidative stress causes the accumulation of β-Amyloid plaques and neurofibrillary tangles, which are shown to

interfere in neuronal function and induce neuronal death (Ang et al., 2010; Ballatore et al., 2007; Hardy & Higgins, 1992).

Similarly, aging is highly associated with skeletal muscle dysfunction. One of the most striking effects of aging on muscle is sarcopenia, a condition described as a progressive decline of muscle mass, strength, and quality (Thompson, 2009). The skeletal muscle is one of the largest consumers of oxygen in the body, which makes it very vulnerable to oxidative stress. During aging, the continuous accumulation of oxidative damage leads to a progressive oxidation of its cellular components, including contractile proteins, causing skeletal muscle dysfunction (Aoi & Sakuma, 2011). Conversely, targeting oxidative stress has shown efficacy at preventing the sarcopenic phenotype in aged mice (Vasilaki et al., 2017). The association of sarcopenia with impaired physical performance, frailty, and increased risk of falls and morbidity is well established in the literature (Doria et al., 2012). Furthermore, sarcopenia is shown to contribute to the development of cardiovascular and metabolic diseases because of its function as an endocrine organ and its involvement in the body's metabolism (Batsis et al., 2014; Ritov et al., 2005; von Haehling, 2018).

Therefore, the better understanding and management of oxidative stress level is very likely to play an important role in the overall aging process, its disease course, and prognosis. Although the nature of the mechanisms underlying aging is at present poorly understood. One of the most accepted theories is the oxidative damage caused by the progressive decline of mitochondrial function with age (Bhatti et al., 2017).

## 4.2 Mitochondrial function, oxidative stress, and the theory of aging

### 4.2.1 Mitochondrial function

Mitochondria are the major producers of reactive oxygen species (ROS), which are a family of highly reactive free radicals generated at the electron transport chain of the inner mitochondrial membrane as a consequence of the aerobic metabolism (Lambert & Brand, 2009). This takes place during oxidative phosphorylation (OXPHOS), the process in which mitochondria convert oxygen and nutrients into adenosine triohosphate (ATP), the cell's

main energy source. The electron transport chain is composed of 5 big complex proteins. ROS are produced as a result of the electrons leakage at complex I and complex III to form superoxide ($O^{2-}$). Subsequently, superoxide is quickly dismutated to hydrogen peroxide (H2O2) (Li et al., 2013). Both superoxide anion ($O^{2-}$) and hydrogen peroxide ($H_2O_2$) are considered strong ROS molecules that can damage macromolecules such as lipids, proteins and DNA (Tsutsui et al., 2011). However, mitochondria are not only responsible for ROS production, but they are equipped with the necessary antioxidant defenses to detoxify them. Under the normal physiological state, the antioxidant system converts ROS into less reactive radicals or completely eliminates them. Therefore, they are of a great importance in maintaining mitochondrial function and protecting the cellular macromolecules. This major role of antioxidant defense is fulfilled by antioxidant enzymes that contribute to the mitochondrial protective function (Sies, 2015). The most important antioxidant enzymes are catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Birben et al., 2012). SOD rapidly catalyzes the conversion of superoxide(O2-) to hydrogen peroxide ($H_2O_2$), which is later metabolized to oxygen and water by either CAT or GPx (Kander et al., 2017).

### 4.2.2 Oxidative stress theory of aging

Mitochondria are negatively affected by aging, leading to an enhanced production of ROS and a diminished antioxidant defense. This consequent imbalance between ROS production and antioxidant defenses leads to oxidative stress (Cui et al., 2012). According to the free radical theory of aging, later termed as oxidative stress theory of aging, aging results from the deleterious effects of ROS causing accumulative structural damage to the macromolecules (lipids, DNA, and proteins). This is followed by tissue and functional losses that is often accompanied by many age-related pathologies (Liguori et al., 2018). Figure 1 describes the cellular mechanism of ROS-mediated oxidative damage in aging. The uninhibited free radicals lead to the lipid peroxidation of poly-unsaturated fatty acids (PUFAs) in membranes, which produce several reactive aldehydes such as trans-4-hydroxy-2-nonenal (4-HNE). As a very reactive aldehyde, and a product of lipid peroxidation, 4-HNE transfers from the membrane into the nucleus and cytoplasm to disrupt the activity of several proteins and DNA. The secondary protein carbonylation

impairs the cellular signal transduction, induces inflammation, and triggers cellular apoptosis (Fedorova et al., 2014). Over time, the accumulation of oxidative damage results in a progressive loss of tissue and organ function, the main mechanism behind aging (Bhatti et al., 2017; Garcia et al., 2017). Due to the high reactivity and short half-life of ROS, investigating the age-related oxidative damage through the direct measurement of ROS is generally considered hard. Therefore, ROS are most commonly tracked by the concentrations of their oxidation target products, including lipid peroxidation end products and oxidized proteins (Dalle-Donne et al., 2006). 4-HNE and protein carbonyls, the products of lipid and protein oxidation, are considered one of the most important markers of oxidative damage in aging studies (Frijhoff et al., 2015).

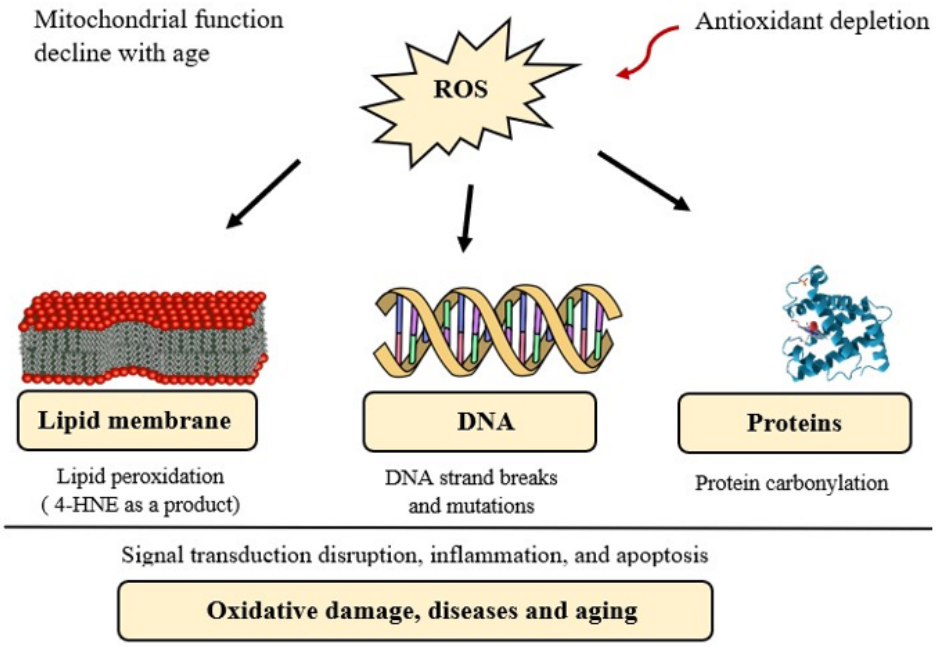


**Figure 1. The cellular mechanism of ROS-mediated oxidative damage of the macromolecules (lipid, DNA, and proteins) in aging.** Arrows indicate induction. Trans-4-hydroxy-2-nonenal (4-HNE). The source of the 3 diagrams (lipid membranes, DNA and protein) is public domain with no attribution required.

### 4.2.3 Hormesis principle

On the other hand, taking the hormesis principle into consideration, sublethal exposure to stressors can induce a response that results in stress resistance (Gems & Partridge, 2008). Research has shown that low concentrations have a beneficial effect (inducing mitochondrial biogenesis, cellular repair, and antioxidant enzyme synthesis), while a massive level of ROS inhibits enzyme activity and causes apoptosis or necrosis (Gems & Partridge, 2008; Giorgi et al., 2018; Słodki & Bogucka, 2019). This positive response to moderate levels of ROS has been termed as mitochondrial hormesis or mitohormesis (Barcena et al., 2018). However, the molecular mechanisms regulating these benefits are largely unknown. Radak et al. proposed that intermittent, brief increases in ROS production (e.g., during exercise training) can cause a slight molecular damage, which further stimulates the oxidative damage repair system (antioxidants synthesis, protein and DNA damage repair). This will protect against a subsequent stronger stress and ROS-associated pathologies, thus slowing down aging (Radak et al., 2005). In fact, the activation of the mitohormetic response is suggested to increase lifespan, which has been shown in different yeast and animal models (Barcena et al., 2018; Musa et al., 2018). Thus, the relationship of ROS to the generated oxidative damage in aging is still not fully understood. In addition, many cofactors such as different sex and exercise capacity have been proposed to play an important role in the body's response to oxidative stress, and thus aging.

## 4.3 Sex-dependent differences in aging

It has been shown that, on average, women in developed countries have an expected lifespan of five years longer than that of men (Popkov et al., 2015). This survival advantage in women has been contributed to the evident sex bias in the manifestation of age-related diseases. Taking cardiovascular diseases into consideration, the leading cause of death, women have a disease onset with a 10-years delay compared to men (Popkov et al., 2015; Radovanovic et al., 2012). In fact, it has been shown that there is a sex-specific cardiac remodeling during aging, in which women could preserve a better cardiac function than men (Ostan et al. 2016). Furthermore, many studies addressing sarcopenia as well have shown a higher prevalence in males in comparison to females (D. R. Bouchard et al., 2009;

Du et al., 2019; Landi et al., 2012). As for neurodegenerative diseases, there is an intriguing asymmetry in the manifestation between sexes, considering that oxidative damage is highly proposed as a common underlying cause. For example, Alzheimer's disease is more likely to affect women, whereas Parkinson's disease is more likely to affect men (Gaignard et al., 2017). Overall, women have been shown to suffer less from most of the leading causes of death compared to men, with the remarkable exception of Alzheimer's disease (Austad & Bartke, 2015).

The specific molecular mechanisms underlying the sex differences in aging remain poorly understood. At present, mitochondrial theory of aging is considered the best agreement explaining the sex-dependent differences in age-induced oxidative damage and its related pathologies (Popkov et al., 2015). To understand these differences, acknowledging the very basic variations between the two sexes at the physiological, cellular, hormonal and genetic level is essential. At the physiological level, men weigh on average 15% more than women. They have higher skeletal muscle mass/body weight ratio (Janssen et al., 2000). At the cellular level, men demand higher oxygen consumption due to the structural differences in muscles, brain, heart, and oxygen capacity of blood. This is assumed to create differences in the mitochondrial biogenesis between the two sexes (Popkov et al., 2015). In addition, estrogen in women plays an important role in inducing mitochondrial biogenesis and preserving mitochondrial function including oxidative stress regulation, cellular proliferation and death (Klinge, 2008). Therefore, estrogen, which also acts as antioxidant itself, contributes to higher cardio- neuro- and skeletal muscles- protective properties in women (Bell et al., 2013; Jung & Metzger, 2016; Lopez-Ruiz et al., 2008; Sullivan et al., 2007). Furthermore, higher expression of genes responsible for mitochondrial network was found in old women compared to old men (Guebel & Torres, 2016). Taken together, all those mentioned multifactorial traits lead to sex-dependent differences in redox hemostasis and the body's response to oxidative stress and aging.

In fact, a human study provided evidence of enhanced oxidative stress in males compared to females of the same age (Ide et al., 2002). Furthermore, a series of studies in a rat model showed that females had lower oxidative stress and mitochondrial dysfunction than males in various tissues (Bhatia et al., 2012; Borras et al., 2003; Brandes & Mugge, 1997; Vina

et al., 2003). Thus, there is a growing number of literature highlighting clear sex-dependent differences in aging, in which women seem to be more resistant to oxidative stress and its related pathologies (Popkov et al., 2015). Another factor that has been shown to play an important role in the mitochondrial biogenesis and function is the genetic predisposition to different aerobic exercise capacity (L. G. Koch & Britton, 2005).

### 4.4 Genetic (intrinsic) exercise capacity and aging

It is well established that low physical fitness is a powerful predictor of premature morbidity and mortality (Blair et al., 1996; Sandvik et al., 1993). Independent of genetics, physical inactivity has been associated with cardiovascular diseases and skeletal muscle dysfunction as well, whereas training showed protective features (Brunjes et al., 2017; Myers et al., 2002). Exercise training is similarly shown to slow down neurodegeneration, by promoting neurogenesis, synaptic plasticity and antioxidant-related pathways (Ang et al., 2010; Gomez-Pinilla, 2008). However, it is important to understand that exercise capacity consists of two components: extrinsic and intrinsic exercise capacity. While the former is acquired through physical activity and other environmental factors, the intrinsic form is genetically determined (C. Bouchard et al., 1999; Kim et al., 2011). Investigating the contribution of intrinsic exercise capacity independently is considered challenging due to the numerous confounding environmental factors. Koch and Britton developed by selective breeding a rat model that was segregated according to its maximal treadmill running capacity into high capacity runners (HCR) and low capacity runners (LCR) (L. G. Koch & Britton, 2001). This animal model offers the possibility to investigate the influence of intrinsic (genetic) exercise capacity separately from the extrinsic form. Rats were tested for their running capacity that included duration, distance, and maximum speed. The best runners were then mated over several generations to create the HCRs line. Likewise, the worst runners were bred analogously to generate the LCRs line. The median age of death between the 2 lines was 24.0 months for LCR rats and 34.7 months for HCR rats, representing a 45% difference in life expectancy (Lauren Gerard Koch et al., 2011).

On the cellular level, LCRs showed a lower expression of proteins that are associated with mitochondrial biogenesis and activity (L. G. Koch & Britton, 2005). On the contrary,

numerous studies found that HCR rats have superior mitochondrial enzyme activities compared to LCR rats (Lessard et al., 2009; Naples et al., 2010; Rivas et al., 2011; Walsh et al., 2006). In fact, LCR rats tend to have increased visceral adiposity, dyslipidemia, increased blood pressure, endothelial dysfunction, and insulin resistance. These features make them develop a phenotype consistent with metabolic syndrome, which is considered a great risk factor for cardiovascular diseases and skeletal dysfunction (Noland et al., 2007; Wisloff et al., 2005). It is also well elaborated in literature that metabolic syndrome can lead to cognitive impair and neurodegenerative diseases like Alzheimer's disease. Two studies revealed evidence of a neurodegenerative process in the aged LCR brains, consistent with those seen in Alzheimer's disease in humans (Choi et al., 2014; Wikgren et al., 2012). Thus, HCR/LCR animal model with its complex phenotypes is considered a great model to investigate the pathogenesis of various systemic diseases during aging, in which oxidative damage is considered the main mechanism behind. This can help us in developing a deeper understanding of the impact of different inherited aerobic and metabolic capacity on the process of aging and its related diseases.

## 5 Aim of the study:

The aim of the current study was to investigate the impact of different intrinsic exercise capacities on the antioxidant defense system and the generated oxidative damage during aging in different sexes.

**The following hypotheses were tested in this study:**

- We hypothesized that HCRs exhibit less oxidative damage with age than LCRs, which is the possible reason for their longer life expectancy.
- We further proposed that there would be sex-dependent differences in the generated oxidative damage with age.

# 6 Materials and Methods:

## 6.1 Materials:

### 6.1.1 Chemicals & Reagents:

The used chemicals and reagents during this work are the following:

| Chemicals | Company |
|---|---|
| $KH_2PO_4$ | Ailied Signal 30407 |
| $K_2HPO_4$ | MERCK A604404 |
| Tris- HCl | Roth 9090.3 |
| EDTA | Roth 8043.3 |
| Glutathione Reductase | Sigma Aldrich G3664 |
| Glutathione reduced | Sigma G4251- 300 MG |
| NADPH | Sigma 93025-1G |
| Cumene Hydroperoxide (80%) | Sigma Aldrich 247502 |
| 30 % $H_2O_2$ | Roth CP26.1 |
| Catalase preparation Aspergillus niger | Sigma C16K3789 |
| Pyrogallol | Sigma P0381- 25 G |
| 37 % HCl. | Roth; X942.1 |
| Triton -X 100 | Sigma, T9284 |
| Tris-HCl | Sigma, T3253 |
| NaCl | Roth, 9265.1 |
| NaF | Sigma, S7920 |
| $Na_4P_2O_7$ | Sigma, S6422 |
| PMSF | Roth, 6367.1 |
| $Na_3VO_4$ | Sigma, S6508 |
| Protease Inhibitor Cocktail-Tablet | Roche, 11697498001 |
| Tris (basic) | Roth, 5429.3 |
| SDS (Sodium Dodecyl Sulfat) | Roth, 2326.2 |
| Dithiothreitol DTT | Roth, 6908.1 |
| Glycerol | Sigma, G7893 |
| Brom phenol blue | Roth, A512.1 |
| Glycine | Roth, T873.2 |
| Methanol | Roth, 4627.5 |
| Tween 20 | AppliChem, A1389 |
| Luminol | Fluka, 9253 |
| Coumarin acid | Sigma, C 9008 |
| $H_2O_2$ | Sigma, 34,988-7 |
| Chemiluminescence reagent for horseradish peroxidase | SERVA, 42582.02 |
| HCl | Roth, K025.1 |
| Ammonium pyrosulfate (APS) | Roth, 9592.2 |
| Isopropanol (2-Propanol) | Sigma, 15,246-3 |

| | |
|---|---|
| Acetic acid | Roth, 3738.5 |
| Coomassie brilliant blue | Serva, 17524 |
| BSA | Sigma, A7906 |
| Protein standard | Sigma, P0834 |
| Coloring reagent concentrate | Biorad, 500-0006 |
| 30% Polyacrylamide | AppliChem, A1672 |
| TEMED | Sigma, T8133 |
| Skimmed milk powder | Roth, T145.2 |
| Serva Visi Plot Standard I | Serva 39260.01 |
| Precision Protein Strep Tactin – HRP Conjugate | Bio-Rad; 161-0380 |

### 6.1.2 Buffers:

| **Buffers** | **Recipe** |
|---|---|
| Lyse buffer | 0,1 M $K_2HPO_4$, 2 mM EDTA, 2 Protease Inhibitor Tablets; pH 7.4; storage by 4 °C for 3 months |
| Tris- buffer | 50 mM Tris- HCl, 0,1 mM EDTA<br>pH 7.6; storage by 4 °C for 2 months |
| GSH- buffer | 0,2 mM NADPH, 9 mM GSH<br>7,5 U/ml Glutathione- Reductase; storage by 4 °C |
| Phosphate buffer | 1 M $KH_2PO_4$, 1 M $K_2HPO_4$<br>pH 7.8; storage by 4 °C for 6 months |
| Pyrogallol | 10 mM HCL, 8 mM Pyrogallol<br>Stored by 4 °C for 1 week |
| $H_2O_2$ | 30 mM $H_2O_2$; freshly prepared for every measurement |
| Protein carbonyl-sample buffer | $Na_2HPO_4$ (1M), $NaH_2PO_4$ (1M), EDTA (0,5M), 0,015g Digitonin, PMSF (0,1mM), Leupeptin (10mM), Pepstatin A (1mM), Aprotinin (1,54mM). Freshly prepared for every measurement |
| Separating/stacking buffers | 2M Tris Hydrochloride, 2 M Tris Basic, 0.4% SDS; pH 8.8. |

| | |
|---|---|
| 10x Running buffer | 144 g Glycine (2M), 10 g SDS (1%), 30g Tris Base (250mM), + dd $H_2O$ up to 1L<br>Stored at 4 °C for 1 month |
| 1x Running buffer | 100ml 10x Stock buffer + 900ml dd $H_2O$ |
| 10x Stock TBS | 87.6g NaCl (1.5M), 60.5g Tris, (500mM) dd $H_2O$ up to 1L; pH 8; stored at -20°C |
| 1x TBS-T | 100ml 10x stock buffer + 900 ml dd $H_2O$. + 1ml Tween 20 (0,1%) |
| 10x Transfer buffer | 30 g Tris Base (250mM), 2 g SDS (1%), 141 g Glycine (2M), dd $H_2O$ up to 1L. |
| 1x Transfer buffer | 100 ml 10x Stock buffer + 200 ml Methanol + 700ml dd$H_2O$ |
| Stripping buffer | 1 M Glycine (37.54 g), dd $H_2O$ up 500ml<br>pH 1.9; stored at -20°C. |
| Blocking buffer | 5% skimmed milk powder in TBS-T |
| Naphthol Blue Black | 200mg Naphthol Blue Black (16.22M), 10 ml Acetic acid (5%), 90 ml Methanol (45%), dd $H_2O$ up 200ml |
| Lämmli 3x | 100 mM Dithiothreitol DTT, 3 mM EDTA, 2,3 mM Tris- HCl, 3 % SDS, 40 % Glycerol, 0,02 % Bromophenol blue |
| Chemiluminescence solution | 1:1000 $H_2O_2$ (30%) + SERVA Chemiluminescence reagent |

### 6.1.3 Antibodies:

| Antibodies | Company | Product number |
|---|---|---|

| | | |
|---|---|---|
| Primary Antibody:<br>Anti-4 Hydroxynonenal antibody | abcam | ab46545 |
| Secondary Antibody:<br>Anti-Rabbit IgG, Horseradish Peroxidase (HRP), linked whole antibody | Amersham pharmacia | RPN4301 |

### 6.1.4 Animal model:

The used animal model, originally from the University of Michigan, Ann Arbor, USA, was specifically selected over 33 generations of females and males rats according to their high (HCR) and low (LCR) intrinsic aerobic running capacity (G. Koch & Britton, 2001). The rats were kept in standard cages with ad libitum access to food and water. The animals were kept at a 12hrs day/12hrs night rhythm at a constant room temperature of 21° C.

## 6.2 Methods:

### 6.2.1 Experimental Design:

Frozen tissues of hearts, brains and skeletal muscles were investigated in each of female and male rats through 4 different groups: young (4 months old) and old (24 months old) rats divided into HCRs & LCRs. Each group contained 6 to 10 samples.

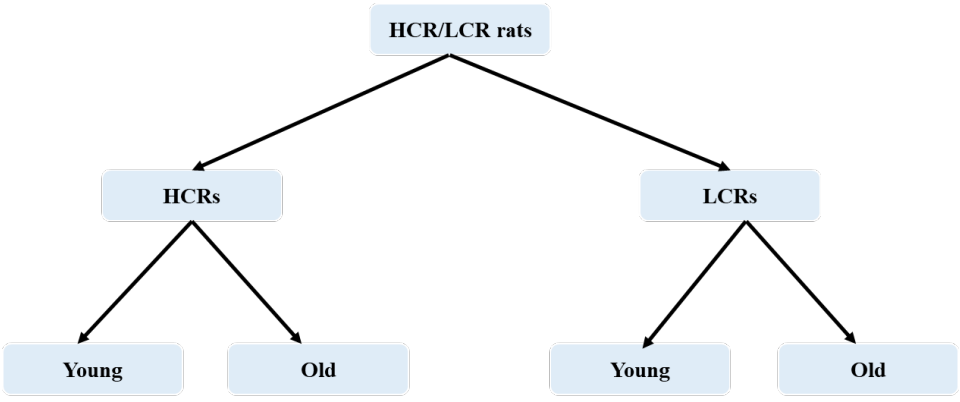


**Figure 1. Flow chart of the experimental design demonstrating the group distribution of the heart / brain / skeletal muscle samples among female and male rats.**

### 6.2.2 Sample Preparation:

The HCR and LCR rats at the ages of 4 or 24 months were anesthetized with thiopental and sacrificed with their organs removed. Thereafter, they were washed in ice-cold NaCl buffer, weighed and stored at -80° C. For this study, the stored frozen tissues of the above-mentioned groups were crushed and powdered under liquid nitrogen and used in measurements.

The sample preparation was carried out according to (Constance Tweedie et al., 2010). 50 mg of the ground tissue were mixed with 500 µl lysis buffer (100 mM $KH_2PO_4$, 2 mM EDTA and 2 tablets protease inhibitor, pH 7.4) and mixed. Thereafter, centrifugation was carried out at 3000* g for five minutes at a temperature of 4° C. The supernatant was collected, transferred into 1.5 ml tubes, and stored at -20° C to be used later for CAT and SOD detection.

The same procedure was conducted again with a centrifugal force of 10,000*g for 15 minutes at a temperature of 4° C to obtain the supernatant used for the measurements of GPx activity (Forstrom et al., 1978; Ursini et al., 1985) and aliquots preparation for HNE Western blotting (Arcaro et al., 2015; Usatyuk et al., 2006). The samples were stored at -20° C when not used at the same day.

### 6.2.3 Quantitative protein determination:

The Bradford photometric method (Bradford, 1976) was used for quantitative protein determination, owing principally to its high sensitivity, perceived linearity, and the speed of analysis (Sapan et al., 1999). The Bradford assay is based on the interactions between basic amino acids residues (arginine, lysine and histidine) with the staining reagent in an acidic environment. This binding results in a color change to the blue form of the dye, depending on the protein concentration of a sample. Prior to the measurement, the prepared samples were diluted with distilled water in a dilution's factors of 1:20 for brains and 1:30 for hearts and skeletal muscles. In order to obtain a standard curve**,** bovine serum albumin (BSA, Sigma P0834) was prepared in varying concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg

/ ml. The staining reagent (BioRad, 500-0006) was diluted 1:5 with distilled water and stored at room temperature until measured. As a blank, to subtract the background, distilled water was used. 5 µl of each of distilled water, standards, and diluted samples were pipetted in triplicates in the corresponding wells of a microtiter plate (MTP, Greiner bio-one, 655101). Immediately afterwards, 200 µl of staining reagent were added using a multichannel pipette. The plate was placed in the photometer for a 3 minutes incubation time followed by the measurement, which was adjusted at a wavelength of 595 nm to detect the spectral shift in color range. Using the standard curve and Lambert-Beer's law, the final protein concentration was calculated and the values were expressed in mg/ml.

### 6.2.4 Catalase activity (CAT):

The detection of catalase activity was conducted according to the method of Aebi (Aebi, 1984), as well as (Banerjee et al., 2010; Weydert & Cullen, 2010) with minor modification. This photometric measurement method is based on a special absorption pattern of hydrogen peroxide at a wavelength of 240 nanometers. Phosphate buffer (1 M $KH_2PO_4$; 1 M $K_2HPO_4$; pH 7.8) was used for dilution. The buffer is stored at 4° C and is stable for 6 months. To control the ongoing reaction, a positive control was prepared using Catalase preparation from Aspergillus niger (Sigma C16K3789).

Pipetting was conducted in triplets in UV sensitive microtiter plates (MTP, Greiner bio-one, 655801) as the following: 150 µl of the phosphate buffer as the blank, 5 µl of the positive control and each of the samples followed by additional 45 µl of phosphate buffer for the latter two. Afterwards 100 µl of $H_2O_2$ (30 mM) were rapidly added using a multichannel pipette. The measurement was carried out immediately using a designed protocol on the photometer (Biotek Synergy 2 Multi-Mode Microplate Reader) measuring interval of 15 seconds, at a wavelength of 240 nm. In this range, the conversion of one micromole of hydrogen peroxide per minute corresponds to a catalase activity of one unit. The molar extinction coefficient of 43.6 M $cm^{-1}$ was used to determine catalase activity. The values were transferred in an Excel spreadsheet and calculated similarly to the catalase activity (Beer, 1852).

### 6.2.5 Superoxide dismutase activity (SOD):

This photometric measurement method is based mainly on the enzyme ability to inhibit the auto-oxidation of pyrogallol at a specific absorption pattern at 420 nm (Ramasarma et al., 2015; Weydert & Cullen, 2010). Normally, the auto-oxidation of pyrogallol in an alkaline environment (pH 7.9-10.6) leads to the formation of quinone compounds which have a specific absorption pattern at 420 nm. In the presence of superoxide dismutase, this reaction is inhibited and the recorded enzyme activity is proportional to the inhibition of autooxidation. The total SOD activity which is the sum of the cytosolic (Cu-Zn-SOD) and mitochondrial (Mn-SOD) activity was analyzed.

Pyrogallol mixture (8 mM) mixed with10 mM HCL was prepared and stored at 4° C for a maximum time of 1 week. The measurement was carried out by pipetting 10 µl of the samples or distilled water (100% values) in triplets into the wells of the MTP (Greiner bio-one, 655101). Subsequently, 280 µl Tris-HCl buffer (0.5 mM, pH 8.5) was added into each of the sample and 100% value wells. As for the blank well, 300 µl of the Tris-HCL buffer was used. After a 10 minutes incubation at 24° C, 10 µl of pyrogallol (8 mM) was added immediately to start the reaction. Detection was carried out for two minutes at an interval of 15 seconds, at a wavelength of 420 nm. The enzyme activity was calculated in units (U) where one unit represents the 50% of the inhibition of the corresponding reaction.

### 6.2.6 Glutathione Peroxidase activity (GPx):

This photometric measurement method detects GPx activity that correlates to the conversion of NADPH to NADP at a special adsorption pattern of 340nm (Paglia & Valentine, 1967; Tappel, 1978). Glutathione exists in reduced (GSH) and disulfide oxidized (GSSG) states. Under physiological state, more than 90% of the total glutathione pool is in the reduced form (GSH). An increased GSSG-to-GSH ratio is indicative of oxidative stress (Lu, 2013).

The measurement is based on the regeneration cycle of the antioxidant enzyme as shown in Fig. X. The regeneration of the reduced state (GSH) is conducted over several reactions catalyzed by glutathione reductase. This regeneration involves the conversion of NADPH into $NADP^+$: $NADPH + GSSG + H_2O \rightarrow 2\ GSH + NADP^+ + H_2O_2$

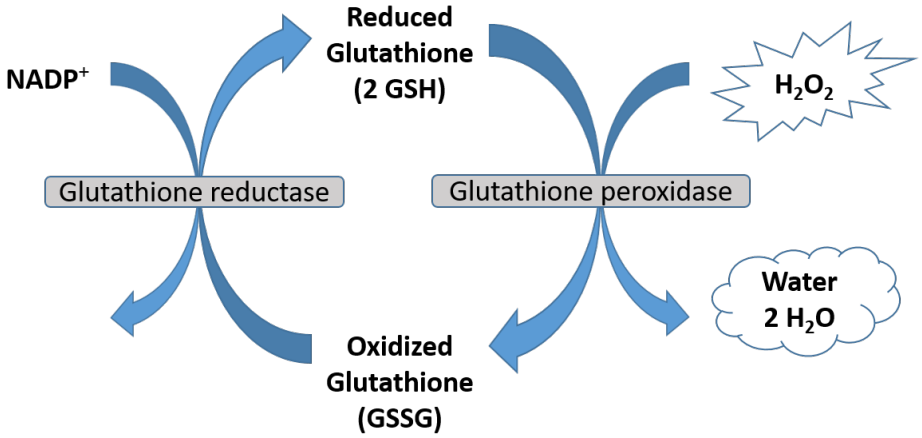


**Figure 2. Regeneration cycle of two glutathione molecules in the reduced state.** The activity of glutathione peroxidase correlates with the consumption of NADPH by glutathione reductase. Glutathione (GSH), oxidized glutathione (GSSG), oxidized nicotinic adenine dinucleotide (NADP), reduced nicotinic adenine dinucleotide (NADPH), hydrogen peroxide (H2O2), hydrogen ion (H +). The cycle involves other substrates and enzymes.

Paglia and Valentine (1967) recognized that the decrease in nicotinamide adenine dinucleotide phosphate (NADPH) levels correlates with GPx activity, where 1 unit corresponds to the conversion of 1 µmol NADPH to NADP per minute at 37 ° C (Tappel 1978). The measurement was carried out using cumene hydroperoxide (1.2 M) as substrate and an assay buffer (GSH 9 mM; NADPH 0.2 mM; Glutathione reductase 7.5 U/ml; pH of 7.6). 15 µl of the undiluted samples were pipetted into triplets in the wells of a MTP (Greiner bio-one, 655101) and 200 µl of GSH buffer were then added. The blank used was 225 µl of the assay buffer. After an incubation period of 10 minutes in the photometer for ten minutes at 37 ° C, 10 µl of cumene hydroperoxides was rapidly added into the wells. The measurement was carried out over two minutes in a measuring interval of 15 seconds at 340 nm. Subsequently, the values were transferred in an Excel spreadsheet and calculated similarly to the catalase activity according to the Lambert-Beer law (Beer,

1852). The molar extinction coefficient of NADPH at 340 nm was used at 0.0062 (1*cm) /µmol.

### 6.2.7 4-HNE western blotting:

Western blotting was used to detect 4-HNE modified proteins according to the standard immunoblotting procedure with some minor modifications.(Mahmood & Yang, 2012; Towbin et al., 1979). The used antibody is highly specific to 4-HNE derived protein adducts (cysteine, lysine, and histidine). It does not cross react with other proteins (Uchida et al., 1993).

*Aliquots preperation:* Following to samples preparation with lyse buffer and their quantitative protein determination with Bradford photometric method (section 2.3), aliquots were prepared for gel electrophoresis. The target protein concentration of the aliquots was 25 µg Protein/15 µl. Based on the sample's protein concentration, the amount of protein necessary was calculated and adjusted with 3× Lammli buffer and distilled water. For protein denaturation, aliquots were heated to 95°C for 10 minutes before use. They were stored at -20 ° C, if not directly measured.

*Electrophoresis:* Polyacrylamide gels including 10% separating and 5% stacking gels of were prepared and stored maximum for 3 days at 4°C prior to electrophoresis. After filling the apparatus with running buffer until 1/3$^{rd}$ of the outer chamber, the gels were loaded with 10 µl aliquots per pocket. One control and a 5 µl of marker (Serva Visi Plot Standard) were used for each gel. The electrophoresis was then initiated to include a running phase of 10 mA/gel for 15 minutes and a separation phase of 25 mA/gel that lasted around 45 minutes.

*Blotting:* After completion of the electrophoresis, the gels were removed from the apparatus and the stacking gel was separated and discarded. The separating gel was washed in transfer buffer. To transfer the proteins from the gel, a transfer membrane (polyvinylidene fluoride - 45 µm pores-Roth) was first activated in methanol for 30 s, washed in distilled water, and then equilibrated again in transfer buffer. This process

involved placing the polyacrylamide gel on the transfer membrane and covering it from bottom and top with 5 filter Whatman papers, previously soaked in transfer buffer. The transfer was carried out for 2 hours at a voltage of 13 V.

*Blocking and Detecting:* After the transfer, membranes were washed for 5 min in wash buffer (TBS-T) and then blocked in 5% milk powder solution for 2 hours at room temperature. This was followed by three 5 min-washings with TBS/T and then an overnight incubation with the primary HNE-antibody (ab46545) with an optimal established dilution ratio of 1:500. On the next day, membranes were again washed three times, 5 minutes each, with TBS/T, and then incubated for 2 hours with secondary antibody (Anti-Rabbit IgG, HRP, RPN4301) with a 1:10000 dilution ratio.

*Evaluation:* LAS 3000 (Luminescent Image Analyzer / Fujifilm) was used to evaluate the membranes after covering each with 3 ml SERVA chemiluminescence solution (3µl $H_2O_2$: 30%) for a better imaging. Finally, the membranes were washed again for 5 min in TBS-T, dyed with naphthol blue-black for 1 hour and then discolored with distilled water. After complete drying in air, membranes were scanned and used for the evaluation of total nonspecific proteins. The program Image J was used to quantify the bands in LAS 3000 images, representing the HNE-bound proteins, and of the normally scanned blue-black images, that represented the total non-specific proteins. The values obtained for the reference protein were used to determine a correction factor, facilitating the comparison between two different membranes.

### 6.2.8 Protein carbonylation (PCO):

Protein carbonylation (PCO) is detected through the derivatization of carbonyl groups by 2,4-Diphenylhydrazone (DNPH). This reaction produces hydrazones that are detected spectrophotometrically at a specific adsorption pattern, (Levine et al., 1990; Reznick & Packer, 1994; Wehr & Levine, 2013).

65 mg of frozen heart, brain or gastrocnemius muscle tissue was weighed and mixed with 1 ml of a freshly prepared assay buffer into 2 ml tubes. The sample tubes were then

homogenized well on ice with at 250U until no tissue pieces were visible anymore. After 15 minutes of rest, the samples were centrifuged at 6000x g for 10 min. The resulting supernatant of each sample was then divided into 2 new sets of tubes: one set served as "sample blanks" and the second was used for the carbonyl measurement. To induce protein precipitation, 450µl 20% TCA was then added to each tube, centrifuged for 2 min at 2000x g, and followed by the removal and the discard of the supernatant. Afterwards, 500µl of DNPH solution or 2.5M HCl was added to carbonyl samples or sample blanks, respectively. Samples were homogenized at 250-300U, until the pellets broke, and then incubated for 15 min in the dark at rest. Subsequently, 500µl 20% TCA was added, samples were vortexed, centrifuged for 2 min at 2000x g, and the resulting supernatant was discarded. The pellets are then washed with 1ml ethanol-ethyl acetate, homogenized, and centrifuged. This step was repeated for 3 times to remove any excess of reactants, with the supernatant discard at end of each step. Finally, 200µl guanidine-HCl was added to the tubes, vortexed and centrifuged prior to measurement.

60 µl of each sample was pipetted as triplicates into a UV sensitive microtiter plates (MTP, Greiner bio-one, 655801). Guanidine-HCl was used in the blank well. The photometric measurement was automatically conducted by a Gen5 Data Analysis software program. The absorption wave was at 370 nm to quantify carbonyl derivates, and at 276 nm to quantify proteins. The obtained values were expressed as the total carbonyl content; unit (mol carbonyl / mol protein).

### 6.2.9 Statistical analysis:

The software Sigma Plot was used for the statistical analysis of the data. The mean values (MV) of the data were calculated and the deviations indicated as standard errors (SE). For the statistical analysis of the obtained values in each of the female and male groups, a two-way ANOVA (HCR / LCR: young / old) was performed, according to the Holm-Sidak method. Statistical outliers were determined by the outlier test according to Grubbs. The significant results were marked with * for the age comparison, and with # for the inherited exercise capacity comparison. The significance was expressed as the following: $P < 0.05$ = * / #, $P < 0.01$ = ** / ##, $P < 0.001$ = *** / ###, and ns = not significant.

# 7 Results:

## 7.1 Oxidative status in Heart:

### 7.1.1 Antioxidant Capacity:

#### 7.1.1.1 Catalase enzyme activity:

Figure 4 shows catalase activity measured in the hearts of young and old HCR and LCR rats in females (A) and males (B). Females showed a similar basal catalase activity between the young HCRs and LCRs rats. A significant increase of enzyme activity with age in both HCR ($P<0.05$) and LCR ($P<0.001$) females was noticed. This increase was bigger in LCR compared to HCR females. Therefore, LCRs showed a higher enzyme activity than HCRs within the old females ($P<0.05$). On the other hand, males showed similar CAT activity between groups with a tendency to decrease with age.

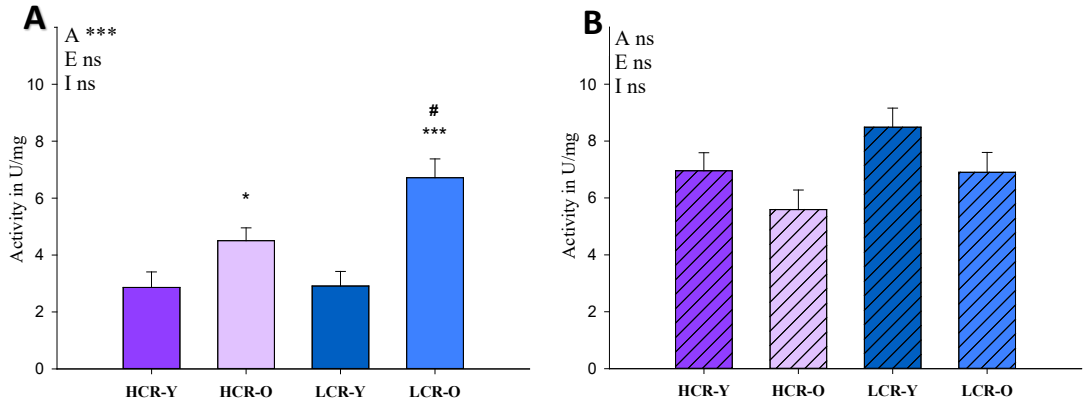


**Figure 3. Catalase activity measured in hearts of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent difference, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

#### 7.1.1.2 Superoxide dismutase and Glutathione peroxidase activities:

Table 1 and 2 demonstrate superoxide dismutase and glutathione peroxidase activities in the hearts of young and old HCR and LCR rats in both females (A) and males (B). In both sexes, SOD showed a tendency to increased activity level in LCRs compared to HCRs in

both young and old groups. As for the GPx activity, all the groups showed relatively similar levels in both sexes.

**Table 1. Superoxide dismutase activity measured in the hearts of 4 and 24 months old HCR and LCR female (A) and male (B) rats.**

| | HCR-Y | HCR-O | LCR-Y | LCR-O | P-A | P-E | P-I |
|---|---|---|---|---|---|---|---|
| **Females** | 353,5 ± 65,1 | 439,8 ± 72,7 | 498,8 ± 56,1 | 609,6 ± 92 | ns | * | ns |
| **Males** | 472,7 ± 70,5 | 449,4 ± 63,1 | 534,9 ± 70,5 | 547,6 ± 51,5 | ns | ns | ns |

Values are presented as mean values ± standard error; enzyme activity measured in U/mg; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

**Table 2. Glutathione peroxidase activity measured in hearts of 4 and 24 months old HCR and LCR female (A) and male (B) rats.**

| | HCR-Y | HCR-O | LCR-Y | LCR-O | P-A | P-E | P-I |
|---|---|---|---|---|---|---|---|
| **Females** | 0,26 ± 0,05 | 0,11 ± 0,06 | 0,15 ± 0,06 | 0,09 ± 0,08 | ns | ns | ns |
| **Males** | 0,12 ± 0,04 | 0,16 ± 0,04 | 0,09 ± 0,03 | 0,12 ± 0,03 | ns | ns | ns |

Values are presented as mean values ± standard error; enzyme activity measured in mU/mg; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

### 7.1.2 Oxidative damage:

#### 7.1.2.1 Protein carbonylation level:

Figure 5 illustrates the protein carbonylation level, an important marker of protein oxidation, in the hearts of young and old HCR and LCR rats in both females (A) and males (B). There was no sex- or phenotype-dependent differences. Both female and males showed similar basal protein carbonyl levels between HCRs and LCRs in the young rats. A strong elevation of protein carbonyls with age was observed in both phenotypes (P < 0.001).

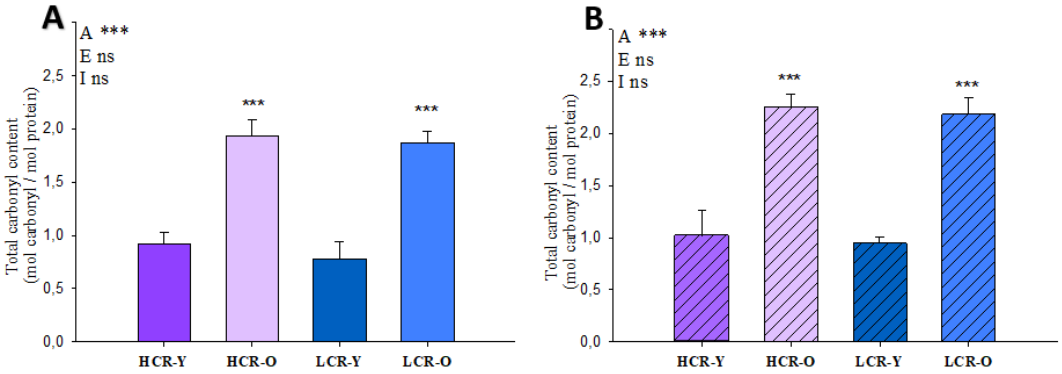


**Figure 5. Protein carbonylation level measured in the hearts of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

### 7.1.2.2 4-HNE level:

Figure 6 presents 4-HNE levels, an important marker of lipid peroxidation, in the hearts of young and old HCR and LCR female (A) and male (B) rats. Both sexes showed similar patterns. Females showed similar basal 4-HNE levels between young HCRs and LCRs. Surprisingly, this level was strongly increased in HCRs ($P < 0.001$) and decreased in LCRs ($P < 0.05$) with age. Similarly, males showed an increase in HCRs ($P < 0.01$) and a tendency to decrease in LCRs with age. Consequently, both females and males had a higher 4-HNE level in old HCRs compared to old LCRs ($P < 0.001$).

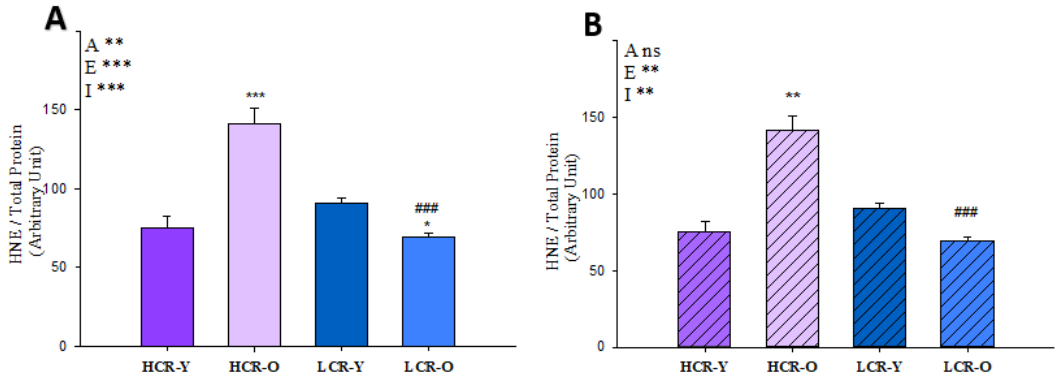


**Figure 6. 4-HNE level measured in hearts of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

## 7.2 Oxidative status in Brain:

### 7.2.1 Antioxidant Capacity:

#### 7.2.1.1 Catalase enzyme activity:

Figure 7 illustrates catalase activity measured in the brains of young and old HCR and LCR rats in females (A) and males (B). Females, basally similar, showed an age-related increase of CAT activity in HCRs (ns) and LCRs ($P < 0.05$). On the other hand, males presented similar CAT activity between all the groups.

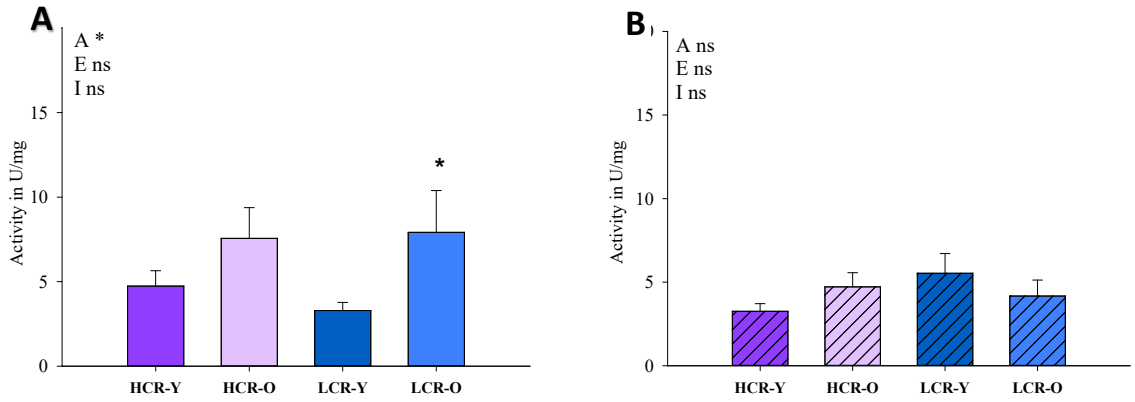


**Figure 4. Catalase activity measured in brains of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

#### 7.2.1.2 Superoxide dismutase activity:

Figure 8 demonstrates superoxide dismutase activity in young and old HCR and LCR rats in both females (A) and males (B). Females did not have any remarkable variations with age or between phenotypes. On the other hand, males presented a similar basal SOD activity between young HCRs and LCRs. This activity showed an age-induced reduction in both HCRs ($P < 0.01$) and LCRs ($P < 0.05$).

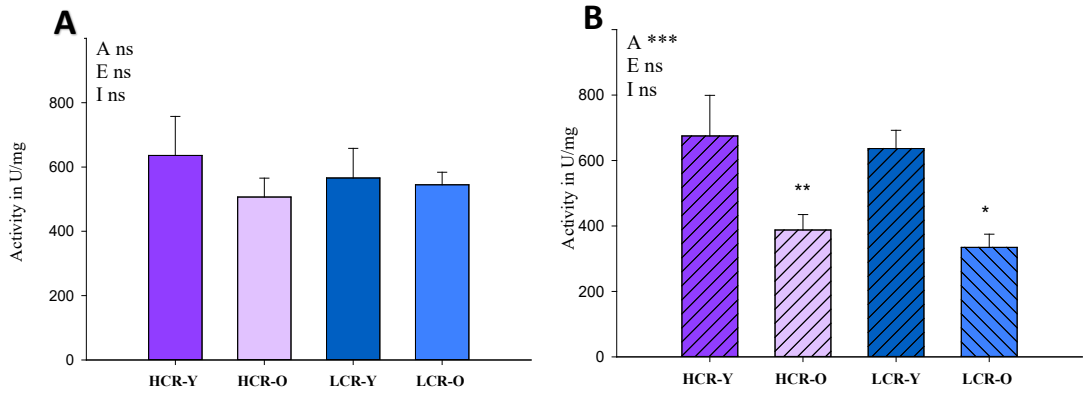


**Figure 5. Superoxide dismutase activity measured in brains of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

#### 7.2.1.3 Glutathione peroxidase activity:

Table 3 lists the values of glutathione peroxidase activity in the brains of young and old HCR and LCR rats in both females (A) and males (B). Both sexes presented similar enzyme activities among all groups.

**Table 3. Glutathione peroxidase activity measured in brains of 4 and 24 months old HCR and LCR female (A) and male (B) rats.**

| | HCR-Y | HCR-O | LCR-Y | LCR-O | P-A | P-E | P-I |
|---|---|---|---|---|---|---|---|
| **Females** | 0,22 ± 0,04 | 0,26 ± 0,04 | 0,19 ± 0,03 | 0,24 ± 0,04 | ns | Ns | ns |
| **Males** | 0,21 ± 0,03 | 0,19 ± 0,03 | 0,2 ± 0,03 | 0,19 ± 0,03 | ns | Ns | ns |

Values are presented as mean values ± standard error; enzyme activity measured in mU/mg; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

### 7.2.2 Oxidative damage:

#### 7.2.2.1 Protein carbonylation level:

Figure 9 reveals the protein carbonylation level in the brains of young and old HCR and LCR rats in both females (A) and males (B). Females showed similar basal PCO levels

between young HCRs and LCRs. A significant age-related increase of protein carbonyls in both HCRs ($p<0.001$) and LCRs ($p<0.001$) was observed. On the other hand, young males presented a significantly higher protein carbonylation in HCRs than in LCRs ($p < 0.05$). Similarly, a significant age-related increase of protein carbonyls in both HCRs ($p<0.01$) and LCRs ($p<0.001$) was observed. There was no difference in the PCO level between old HCRs and LCRs in both sexes.

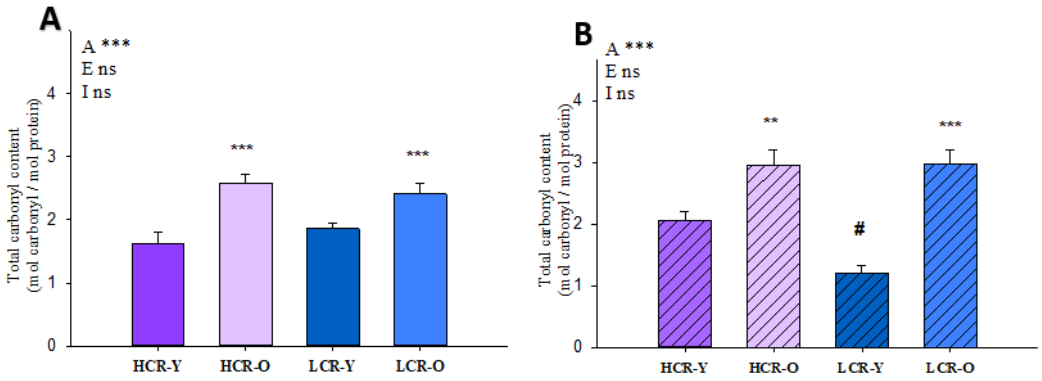


**Figure 9. Protein carbonylation level measured in brains of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

#### 7.2.2.2 4-HNE level:

Figure 10 presents 4-HNE levels measured in the brains of young and old HCR and LCR female (A) and male (B) rats. Both sexes, basally similar, showed a strong age-induced elevation of 4-HNE levels in both HCRs ($p<0.01$) and LCRs (females: $p<0.05$; males: $p<0.001$). No significant variations between the phenotypes were noticed in both sexes.

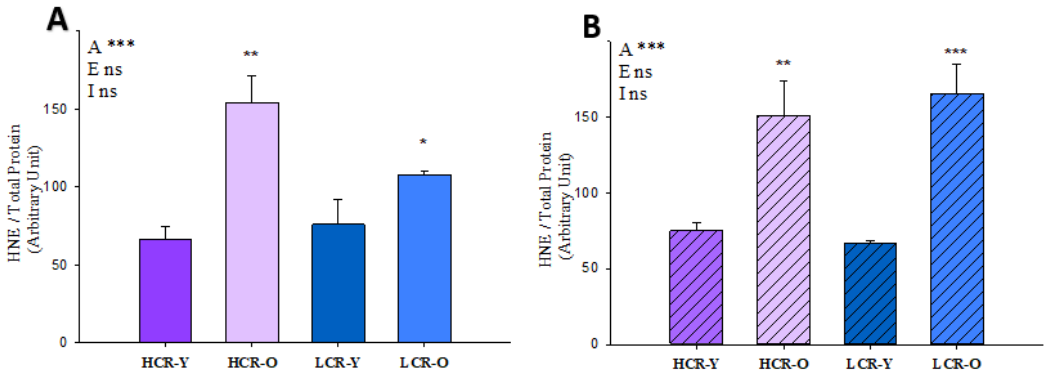


**Figure 10. 4-HNE level measured in brains of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

## 7.3 Oxidative status in Skeletal Muscle:

### 7.3.1 Antioxidant Capacity:

#### 7.3.1.1 Catalase enzyme activity:

Figure 11 shows catalase activity measured in gastrocnemius muscles of young and old HCR and LCR rats in both females (A) and males (B). Both sexes showed similar basal CAT levels in the young groups. A remarkable age-related elevation of CAT activity was only observed in HCRs ($p < 0.001$) of both sexes. LCRs maintained a relatively unchanged activity with age. This led to a significantly higher CAT activity in HCRs than in LCRs among the old groups ($p < 0.001$).

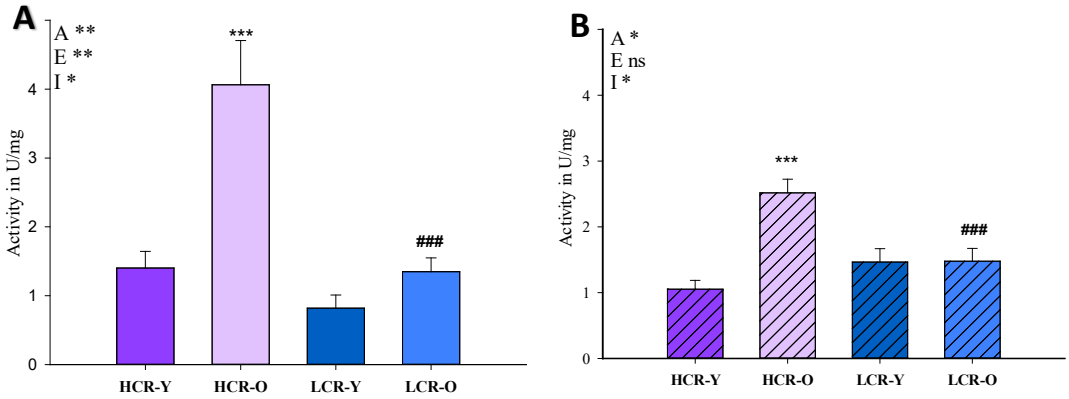


**Figure 6. Catalase activity measured in gastrocnemius muscles of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

### 7.3.1.2 Superoxide dismutase and glutathione peroxidase activities:

Table 4 and 5 demonstrate superoxide dismutase and glutathione peroxidase activities in the gastrocnemius muscles of young and old HCR and LCR rats in both females (A) and males (B). SOD showed a tendency to be higher in HCRs among female rats and in LCRs among male rats. As for GPx, similar enzyme activities were observed among all groups of both sexes.

**Table 4. Superoxide dismutase activity measured in gastrocnemius muscles of 4 and 24 months old HCR and LCR female (A) and male (B) rats.**

| | HCR-Y | HCR-O | LCR-Y | LCR-O | P-A | P-E | P-I |
|---|---|---|---|---|---|---|---|
| **Females** | 320,2 ± 43,4 | 310,2 ± 38,3 | 251,8 ± 38,3 | 252 ± 51,3 | ns | ns | ns |
| **Males** | 269 ± 25,1 | 298,2 ± 30,7 | 416,4 ± 39,6 | 363,4 ± 29,2 | ns | * | ns |

Values are presented as mean values ± standard error; enzyme activity measured in U/mg; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

**Table 5. Glutathione peroxidase activity measured in gastrocnemius muscles of 4 and 24 months old HCR and LCR female (A) and male (B) rats.**

| | HCR-Y | HCR-O | LCR-Y | LCR-O | P-A | P-E | P-I |
|---|---|---|---|---|---|---|---|
| **Females** | 0,01 ± 0,004 | 0,02 ± 0,004 | 0,028 ± 0,007 | 0,028 ± 0,008 | ns | ns | ns |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Males** | 0,028 ± 0,005 | 0,01 ± 0,005 | 0,014 ± 0,003 | 0,028 ± 0,006 | ns | ns | ns |

Values are presented as mean values ± standard error; enzyme activity measured in mU/mg; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

### 7.3.2 Oxidative damage:

#### 7.3.2.1 Protein carbonylation level:

Figure 12 illustrates the protein carbonylation level measured in gastrocnemius muscles of young and old HCRs and LCRs in female (A) and male (B) rats. Both sexes presented similar basal protein carbonylation levels in young HCRs and LCRs. A strong elevation of protein carbonyls with age in HCRs ($p<0.001$) and LCRs (females: $p<0.001$; males: $p<0.01$) was present. Furthermore, old females showed a significantly higher level of protein carbonylation in LCRs than in HCRs ($p<0.01$).

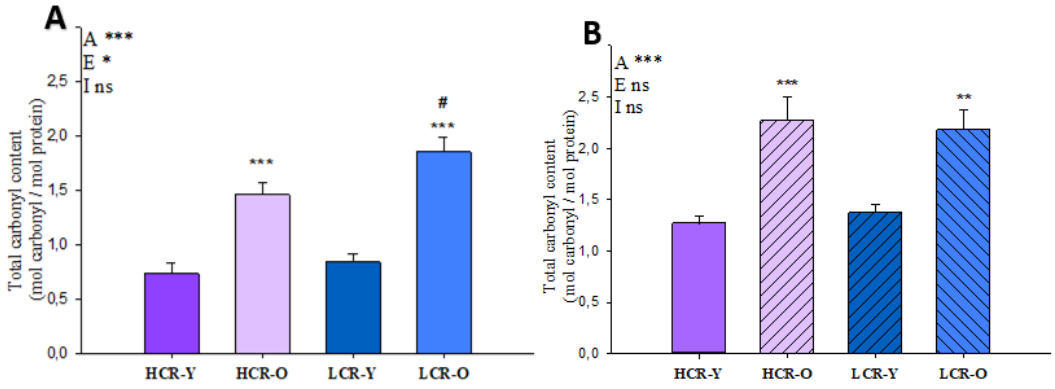


**Figure 12. Protein carbonylation level measured in gastrocnemius muscles of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

#### 7.3.2.2 4-HNE level:

Figure 13 demonstrates the measured 4-HNE level in gastrocnemius muscles of young and old HCR and LCR females (A) and males (B). Both sexes showed no significant change in 4-HNE level with age. Females presented similar 4-HNE levels among all groups. On the

other hand, males showed a higher 4-HNE level in the young HCRs compared to the young LCRS ($p < 0.01$). This difference disappeared among the old males.

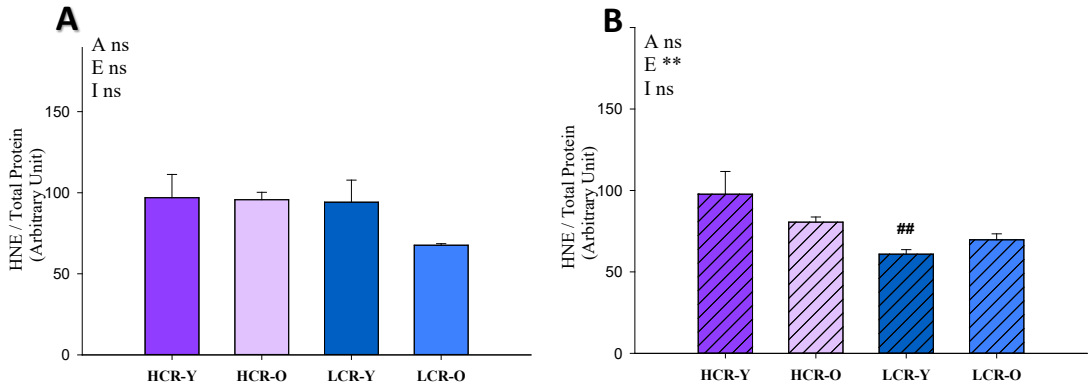


**Figure 7. 4-HNE level measured in gastrocnemius muscles of 4 and 24 months old HCR and LCR female (A) and male (B) rats.** Mean values ± standard error; n =6-10; A (*): age-dependent differences, E (#): intrinsic exercise capacity (phenotype) dependent differences, I: interaction; the significance was expressed as the following: */# = P <0.05, **/## = P <0.01, ***/### = P <0.001, and ns = not significant.

## 8 Discussion:

We aimed to investigate the role of different intrinsic exercise capacities on the antioxidant defense system and the generated oxidative damage during aging in both sexes. In this work, it could be shown that intrinsic exercise capacity did not have an evident impact on the generated oxidative damage with age in heart and brain. Higher protection of HCRs in the skeletal muscle due to a higher antioxidant capacity with age could be assumed. Furthermore, both sexes presented similar patterns of oxidative damage during aging, despite of a higher antioxidant potential in female hearts and brains.

One of the recently proposed factors showing a strong effect on life-expectancy is the intrinsic (genetic) exercise capacity (L. G. Koch & Britton, 2005). Through the HCR/LCR rat model, we could investigate for the first time the effect of different intrinsic exercise capacities on the generated oxidative damage markers in the context of aging. In heart, we found surprisingly that LCRs exhibited a higher antioxidant capacity and a lower lipid peroxidation marker (4-HNE) with age, compared to HCRs. The greater antioxidant capacity in old LCRs was represented by a higher CAT enzyme activity in females as well as a tendency to higher SOD activity in both sexes. Similar findings of higher CAT and SOD levels with age in LCRs was obtained previously by our working group (Schenkl, 2018). Despite the lack of aging-focus, two previous studies conducted proteomic analysis on the hearts and livers of adult HCRs and LCRs. Interestingly, they found that LCRs exhibit higher expression of CAT enzyme and of 4-HNE as well in both hearts (Burniston et al., 2011) and livers (Thyfault et al., 2009). The authors argue that enhanced expression of catalase in LCRs is due to a higher level oxidative stress in LCRs, compared to HCRs. Nevertheless, this is less likely to serve as an explanation in our study, because LCRs showed lower (4-HNE) or similar (PCO) oxidative damage markers with age compared to HCRs. Discrepancy in findings can be highly related to the differences in tissue type as well as in animal ages. Another unexpected finding of this working group was that LCRs survive longer under pressure-overload-induced heart failure in comparison to HCRs (Schenkl, 2018). A strong contributor for that was assumed to be the greater antioxidant capacity in LCRs as a results of their higher exposure to moderate amounts of ROS. Taking the hormesis principle into consideration, moderate levels of ROS can cause a slight molecular damage, which induces positive effects such as antioxidant enzyme syntheses

and cellular repair mechanisms (Radak et al., 2005). Assuming that LCRs are more exposed to moderate ROS production with age might not only explain their higher antioxidant capacity in this work, but also their lower lipid peroxidation marker (4-HNE) as a result of an increased cellular repair. Two studies using this rat model found that LCRs carry similar or even lower risk for contractile dysfunction and myocardial ischemia, compared to HCRs (Høydal et al., 2013; Hussain et al., 2001). The authors could not also support the cardio-protective effect of the high inherited exercise capacity. They further concluded that the protective effect of an enhanced inherited aerobic capacity in HCRs is only acquired as a response to training. Physical fitness and exercising, independent of the genetic predisposition, are shown to be highly protective against age-related pathologies including cardiovascular diseases (Blair et al., 1989; Roh et al., 2016; Warburton et al., 2010). Based on our results, genetic predisposition alone to high aerobic running capacity is not associated with decreased oxidative damage in the aging heart.

Similarly in brains, we found that LCRs had a higher CAT activity than HCRs in old females. Both oxidative damage markers, however, were similarly elevated during aging in both phenotypes. Evidence of recent studies using this rat model showed that LCRs carry a higher risk profile for neurodegenerative diseases. Despite the lack of focus on aging, Sarga et al. showed that adult male HCRs had a better spatial memory, which was related to decreased protein carbonyl levels and DNA damage in the brain, when compared to LCR rats (Sarga et al., 2013). Interestingly, they noticed that after exercise training, oxidative damage markers were unexpectedly higher in HCRs, yet they still performed better in comparison to LCRs. Furthermore, Choi et al. examined aged rats (25 months old) and found that old LCRs exhibit many features of Alzheimer's disease, including mitochondrial abnormalities, neuronal loss, decreased hippocampal volume as well as impaired cognitive function. Surprisingly, they observed that there were no significant differences in mitochondrial DNA damage between LCR and HCR rats (Choi et al., 2014). This raises the question whether oxidative damage markers are enough to reflect the evident differences in cognitive function between the two phenotypes. Based on our results, both HCR and LCR phenotypes showed similar patterns of age-related oxidative damage in brain tissue. However, this does not rule out variable aging processes in the brains of HCRs and LCRs. In the light of the previously discussed studies, we therefore assume that

differences in oxidative damage markers might not be relevant to the evaluation of cognitive function and neurodegenerative rate between the two phenotypes.

In skeletal muscles, we could show that high inherited exercise capacity was associated with a higher antioxidant potential in aged rats. HCRs presented a significantly higher CAT activity than LCRs in the old rats. The benefits of antioxidants in declining skeletal muscle aging and preventing the negative effects of ROS is well established (Brioche & Lemoine-Morel, 2016; Steinbacher & Eckl, 2015). As for oxidative damage markers, HCRs showed a lower level of PCO in comparison to LCRs among old females. Using this rat model, Tweedie et al. is so far the single study investigating antioxidants in the skeletal muscle. Similar to our results, they showed that HCRs have a higher antioxidant capacity compared to LCR rats. Interestingly, they found that HCRs generate higher ROS levels despite of lower DNA damage and higher antioxidant level. This was related to the previously discussed hormosis principle, in which moderate ROS levels in HCRs can promote beneficial cellular adaptations including higher antioxidants and cellular repair (C. Tweedie et al., 2011). In the same context, Radak et al. proposed that intermittent increases in ROS production can stimulate the oxidative damage repair system including the proteasome complex, which is responsible for the degradation of oxidatively modified proteins and their replacement (Radak et al., 2005). Thus, it can be assumed that HCRs are exposed to a moderate production of ROS in the skeletal muscle, which explains the higher CAT production as well as the lower protein oxidation marker (PCO). In addition, the enhanced physical performance and intrinsic motoric skills in HCRs lead to a higher resistance against sarcopenia, which is determined by the presence of both low muscle mass and low muscle function (Cruz-Jentoft et al., 2010). On the other hand, sarcopenia tends to be associated with obesity. This association is termed as sarcopenic obesity, in which adipose tissue secretes bioactive molecules and high levels of ROS, causing further metabolic complications including sarcopenia (Lefranc et al., 2018). This suggests a higher susceptibility of LCRs to sarcopenia due to their predisposed adiposity in their phenotype. This could also explain the higher levels of PCO in LCRs among old female rats. In the light of the previously discussed findings and based on our results of higher antioxidant capacity in HCRs, we assume that HCRs are more protected against age-related oxidative damage in skeletal muscle.

In the HCR/LCR rat model, previous publications have not so far investigated sex-differences in antioxidant capacity and oxidative damage markers during aging. However, several studies using different rat models showed that females are less prone to oxidative stress in hearts (No et al., 2018), brains (Borras et al., 2003), microvessles (Dantas et al., 2004), aortic endothelium (Brandes & Mugge, 1997), and kidneys (Bhatia et al., 2012) in comparison to males. In this work, both sexes presented similar elevation patterns of oxidative damage markers with age, despite that females had a higher antioxidant capacity with age in the hearts and brains. This was represented by an age-dependent increase of CAT enzyme activity in hearts and brains of females. In contrast, males showed unchanged CAT activity and a strong reduction of SOD activity with age in brains. There is a lack of uniform consensus in literature on the relevance of antioxidant enzymes in cardiac tissue and their significance in different sexes (Barp et al., 2002; Colom et al., 2007). However, the loss of antioxidant defenses is shown to be highly involved in the aging process of the brain (Gaignard et al., 2017). In a series of studies addressing the brain tissue in Wistar rats, females showed higher antioxidants and less mitochondrial DNA damage compared to males. The authors argued that their findings are consistent with the oxidative stress theory of aging, which explains the survival advantage in female rats (Borras et al., 2003; Vina et al., 2003). Further, previous publications presented similar findings to ours of an age-dependent reduction of SOD in the brain of male rats (Ehrenbrink et al., 2006; Samarghandian et al., 2015; Tsay et al., 2000). In fact, SOD has been shown to be as one of the major targets of oxidative damage in neurodegenerative diseases (Gonos et al., 2018; Semsei et al., 1991), which could be even used as a prognostic marker (Flynn & Melov, 2013). In conclusion, both sexes seem similarly exposed to the generated oxidative damage with age in this rat model. However, our findings imply that males have a diminished antioxidant capacity with age. This could potentially contribute to an accelerated aging in the male brain.

Altogether, the oxidative stress theory of aging is based on the hypothesis that aging is caused by the accumulation of oxidative damage to macromolecules, which consequently contributes to several age-related pathologies (Betteridge, 2000; Liguori et al., 2018). Consistent with the above described hypothesis, we found in this study a remarkable increase of oxidative damage markers with age in all groups. This was represented by the

elevation of PCO in all tissues and that of 4-HNE in brains and selectively in hearts. We therefore could demonstrate an apparent association between oxidative damage and aging, which potentially supports the oxidative stress theory of aging. Nevertheless, our results showed no relevant variations of oxidative damage markers between different sexes and phenotypes. This, however, does not rule out other possible distinctive features. It is important to note that some types of macromolecular damage (to DNA, proteins or lipids) are reversible because of the repair mechanisms. Thus, the functional relevance of this damage could be also dependent on the capacity to repair it, which is often not measurable (Robert & Bronikowski, 2010). According to hormesis principle, moderate ROS production might be involved in stimulating this oxidative damage repair system (Radak et al., 2005). Thus, despite that our results suggest similar patterns of oxidative damage during aging in different phenotypes and sexes, other possible differences in the tolerance and repair capacity might be assumed.

*Limitations of the study:*

The results of our study may have some limitations in measurements. Most notably, our experimental design did not control for variations in the estrus cycle of female rats, which could affect the circulating estrogen levels over a period of several days. Investigations of mitochondrial DNA damage, direct ROS emission, and mitochondrial capacity might have increased the understanding of our results.

## 9 Conclusion:

We could show in this work that oxidative damage markers increased with age, which potentially supports the oxidative stress theory of aging. Genetic predisposition to high aerobic capacity was, however, not associated with a decreased oxidative damage during aging in hearts and brains. In skeletal muscles, a higher protection in the HCRs could be assumed due to the higher antioxidant capacity with age. Furthermore, both sexes presented similar patterns of oxidative damage during aging, despite of a higher antioxidant potential in female hearts and brains.

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# 11 Appendix

## 11.1 List of Figures

## 11.2 List of Tables

## 11.3 Acknowledgement

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## 11.4 Declaration of honor

I hereby declare that I am aware of the doctoral regulations of the Medical Faculty of the Friedrich Schiller University, and that I have authored this thesis independently without using other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

The following individuals have supported me with the selection and evaluation of the presented work:

Prof. Dr. med. Torsten Doenst

PD Dr. rer. nat. habil. Michael Schwarzer

Estelle Heyne.

No further individuals were involved in the preparation of the presented thesis with respect to content and materials. In particular, I did not receive paid assistance or consulting services from PhD consultant or others.

Some results of this work were included in the thesis of Estelle Heyne. This work has to date not been submitted domestically or abroad in the current or in a similar version to any other examination board.

I declare on my honor that I have told the truth to the best of my knowledge and have not concealed anything. I am also aware that the respective work can be considered as a "fail" in the event of a false declaration.

_____

Jena, Rita Musleh

## 11.5 Curriculum vitae

**Personal Data**

| | |
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| Name: | Rita Musleh |
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**Education**

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**Scholarships:**

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