A complex dietary supplement ameliorates aspects of aging

Aspects of metabolism and energy use in aging as impacted by a complex dietary supplement.

By

Sarthak Matravadia, B.Sc. (Hons.)

A Thesis submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2011) McMaster University
(Biology) Hamilton, Ontario

TITLE: Aspects of metabolism and energy use in aging as impacted by a complex dietary supplement.

AUTHOR: Sarthak Matravadia, B.Sc. (Hons.) (McMaster University)

Supervisor: C. David Rollo, Ph.D., Professor (McMaster University)

Co-Supervisor: Gianni Parise, Ph.D., Professor (McMaster University)

Co-Supervisor: Grant McClelland, Ph.D., Professor (McMaster University)

Number of Pages: xii, 137

Abstract:

Aging involves the progressive decline of physical performance and effective metabolic regulation. To date, dietary interventions to slow this deterioration have shown limited success. I tested the effectiveness of a complex dietary supplement (that targets five key mechanisms of aging) for ameliorating age-related declines in physical activity, metabolism and energetic efficiency in mice. Supplemented mice maintained youthful levels of daily physical activity in old age, compared with a progressive decline in untreated controls. The diet also influenced aspects of metabolic rate, as supplemented mice showed age-related increases in fasting oxygen consumption and respiratory quotient compared to declines in these biomarkers in untreated mice. Furthermore, oxygen consumption over 24-h was significantly lower in supplemented mice in spite of being more active than untreated mice. Taken in conjunction with higher resting respiratory exchange ratios across age, this suggests that supplemented mice may utilize more carbohydrate than lipid as an energy substrate and they may express increased metabolic efficiency. These results hold promise for augmenting youthful athleticism and extending geriatric functionality. I also assessed the impact of the supplement on age-related changes in biomarkers of oxidative stress in heart and kidney samples from normal (Nr) and transgenic (Tg) mice that over-express growth hormone. Measures of whole-tissue H_2O_2 in the heart showed no significant changes in Nr or Tg mice, but catalase activity was ~33% higher in supplemented Nr and Tg compared to untreated controls. Kidney tissue from Nr mice showed significant and opposite age-related trends of H_2O_2, increasing in supplemented mice and decreasing in untreated controls, however,

no changes were observed in Tg mice. Catalase activity in kidney tissue remained unchanged in both genotypes regardless of diet. Furthermore, the ratio of reduced to oxidized glutathione was 43% higher in urine from older (>12 months-old) supplemented mice, indicative of substantially lower whole-body oxidative status. Lastly, older supplemented mice showed improved whole-body glucose tolerance compared with untreated counterparts. These results confirm that the supplement reduces aspects oxidative stress and improves insulin sensitivity, two of the key design criteria for formulating the supplement. This work represents proof of principle that complex dietary supplements can extend functional capacities associated with metabolism and energetic efficiency into older ages.

Acknowledgements:

This thesis would not have been possible without the unbelievable mentorship, support and knowledge of my supervisor, Dr. C.D. Rollo. His passion and dedication have been a continuing source of inspiration in my journey as an aspiring researcher. By learning from his advice and life experiences I have also grown as a person, for which I am inexplicably grateful. I also thank Vadim Aksenov being an incredible mentor and for taking me on as a volunteer 5 years ago, as this was the starting point of my journey with the Rollo lab. Through his patience, support and friendship I have gained valuable perspective and understanding that will help me succeed in life.

I thank Dr. G. Parise for his helpful input and direction as a member of my supervisory committee. In addition, I thank Dr. G.B. McClelland for facilitating several of my experiments and opening the doors to the McClelland lab to help me complete my investigations. It has been a great experience working with both of you.

To my parents, words cannot express my gratitude for the unconditional support, patience and understanding. Without their motivation the completion of this thesis would not have been possible. They are my inspiration and I hope to keep making them proud.

To my friends and colleagues, it has been a pleasure working with all of you and I wish each of you the very best in the times ahead.

TABLE OF CONTENTS

List of Figures **Page**

LIST OF TABLES

LIST OF ABBREVIATIONS

ATP – adenosine triphosphate

CAT - catalase

CuZn-SOD – copper zinc superoxide dismutase

DSP – complex dietary supplement

FRTA – free radical theory of aging

GH – growth hormone

GHRH – growth hormone releasing hormone

GHRKO – growth hormone receptor knockout

GPx – glutathione peroxidase

GSH – reduced glutathione

GSSG – oxidized glutathione

IGF-1 – insulin-like growth factor-1

Mn-SOD – manganese superoxide dismutase

Nr – normal wild-type mice

RER – respiratory exchange ratio

ROS – reactive oxygen species

RQ – respiratory quotient

SOD – superoxide dismutase

SpA – spontaneous activity levels

Tg – transgenic growth hormone mice

CHAPTER ONE: INTRODUCTION

1.1 Revisiting the free radical theory of aging

Aging encompasses complex degenerative processes impacting multiple physiological systems. Although our understanding of these processes is often rudimentary, recent advances have given investigators the impetus to uncover evidence that allows for critical assessment of traditional aging theories. While disproval of any theory is by necessity a fundamental goal of research programs, to ensure sustainable progress there must be a concomitant development of newer ideas and hypotheses. Here I will revisit a classic aging paradigm and review evidence that supports and challenges its predictions.

The 1950's saw the birth of Harman's free radical theory of aging (FRTA), which has dominated modern day aging research. The general theme is that aging is a manifestation of the lifelong accumulation of damage in mitochondrial and cytosolic constituents caused by harmful reactive oxygen species (ROS), an intrinsic byproduct of mitochondrial energy metabolism (Harman, 1956). Over time cellular integrity progressively declines, making it increasingly difficult to maintain optimal functionality of genetic and metabolic processes. More than ever, it is accepted that aging is a multidimensional process attributed to no single factor, yet most renowned aging theories including the FRTA are centered on a single physiological cause. Although numerous exceptions have weakened the FRTA's foundation, an enormous wealth of information has been accumulated over five decades that generally supports predictions of the theory.

There are several related hypotheses that have indeed been supported through various experimental models, but should not be confused with the central hypotheses that ROS are the cause of aging. An example of a related hypothesis would be the notion that mitochondrial oxidative damage accumulates with age, or that oxidative damage eventually compromises functional capacity over time. Here, some of the recent evidence that has brought Harman's FRTA under critical evaluation will be reviewed.

1.2 Genetic manipulation – controversy with the code of life

If ROS are the cause of aging, then obtaining an extension of lifespan by reducing ROS production or ROS-associated damage (i.e. through antioxidants) would support the FRTA. Genetically engineered rodent models that over-express key antioxidant enzymes such as copper-zinc superoxide dismutase (CuZn-SOD) and manganese superoxide dismutase (Mn-SOD) showed great promise. Rollo et al. (1996) predicted a priori that the transgenic growth hormone mouse that expresses accelerated aging would also express elevated free radical processes. Free radical levels and damage in brain and heart proved to significantly predict longevity. However, there have also been contradictory findings. For example, a transgenic mouse model with a 2-5 fold over-expression of CuZn-SOD in all tissues failed to extend lifespan (Chen et al., 2003; Huang, Carlson, Gillespie, Shi, & Epstein, 2000). In addition, despite protection against oxidative stress, mice over-expressing Mn-SOD, an important mitochondrial antioxidant enzyme, also failed to show any impacts on longevity (Jang et al., 2009). These results are in disagreement with the FRTA because it would be expected that any reductions in ROS, particularly those

achieved through constitutive over-expression of important antioxidant enzymes, should translate into an extended lifespan. However, this does not imply that normal levels of antioxidant expression do not play a role in determining lifespan.

In general, it is held that mitochondrial free radical generation is more important than variation in antioxidant systems. One might ask why these systems are present if they don't do anything important. The first strong support for the FRTA was the discovery of superoxide dismutase in the early 1960's as predicted by the theory (Harman, 1956). There are examples where increases in antioxidant enzymes in various tissues failed to extend lifespan. Schriner et al. (2005) demonstrated a remarkable 17% extension in lifespan in mice over-expressing catalase targeted to mitochondria (Schriner et al., 2005). However, further investigation revealed that this may have been in part due to a genetic artifact associated with the engineering of these mice, and therefore raises doubts over credibility (Schriner et al., 2005). It was also observed that catalase was differentially over-expressed in tissues (i.e. substantial increase in heart but no change in kidney) (Schriner et al., 2005). The development of transgenic rodents over-expressing different combinations of principle antioxidant enzymes such as Mn-SOD, CuZn-SOD and catalase also failed to extend longevity in mice (Perez et al., 2009).

It is important not to overlook the value of genetic knockout models, where single or double combinations of antioxidant enzymes have generated interesting results. CuZn-SOD knockout lines are both viable and grow otherwise normally, but exhibit signs of extremely elevated oxidative stress, i.e. plasma F_2-isoprostanes (Muller et al., 2006; Sentman et al., 2006). This phenotype results in a ~30% decrease in lifespan, but also

showed susceptibility to pathologies not usually seen in background C57BL/6 such as hepatocellular carcinoma (Sentman et al., 2006). Mn-SOD heterozygous knockout mice also appear to grow to adulthood normally, but are highly susceptible to oxidative stress through an accumulation of damage in both nuclear and mitochondrial genomes along with elevated protein carbonyls (Van Remmen et al., 1999; Van Remmen et al., 2001; Williams et al., 1998). Glutathione peroxidase (GPx) is an important cellular antioxidant found in most tissues, with the primary isoform being GPx1. Mice with complete knockout of GPx1 not only develop to adulthood relatively normally, but also showed no loss in longevity despite increased susceptibility to oxidative stress via H_2O_2 (Y. Zhang et al., 2009).

The evidence presented in these studies converges upon the idea that ROS alone may not fully explain aging. The mitochondrial DNA mutator mouse is a key example, where high mitochondrial mutation rates produce symptoms of aging, but ROS stress appears to be absent. This strongly implies an energetic basis for aging independent of ROS (Edgar & Trifunovic, 2009). Likewise, it appears that the ubiquitous or tissue-specific over-expression of single and multiple antioxidant enzymes do not predict lifespan. However this alone does not necessarily dismiss the involvement of ROS in aging. Similarly, the use of genetically engineered over-expression/knockout models are valuable in testing the predictions of the FRTA, but findings must be interpreted with caution as physiological processes relevant to aging are complex and require a multi-dimensional approach. Although recent evidence appears to contradict the FRTA in certain contexts, the importance of endogenous antioxidant enzymes in the maintenance

of health cannot be rebuffed. This is particularly true when the role of ROS as intracellular signaling molecules is considered; therefore the goal should not be to eliminate ROS entirely, but rather promote maintenance of regulated functional levels. The complexity of research endeavors grows as newer aging theories offer promising avenues of future study, such as the role of a temporal framework linking growth and stress functions during specific sleep windows (Rollo, 2010).

1.3 Aging paradox: increased oxidative stress equals increased lifespan?

To add to the controversy surrounding the FRTA, studies using long-lived rodent models sometimes show elevated markers of oxidative stress than short-lived counterparts. For example, the Ames dwarf mouse is both growth hormone (GH) and insulin-like growth factor (IGF) deficient and exhibits elevated mitochondrial ROS production in cardiovascular tissue (Csiszar et al., 2008). However, these dwarf mice also show elevated stress resistance through increased forkhead box-O (FOXO) transcription factor activity, which may lower oxidative stress in other tissues (Murakami, 2006). In addition, the naked-mole rat is another exceptionally long-lived model that appears at odds with Harman's FRTA (Andziak, O'Connor, & Buffenstein, 2005; Andziak et al., 2006). Naked-mole rats have higher levels of oxidative stress than some short-lived rodent models, including elevated oxidative damage to DNA, protein and lipid (Andziak et al., 2005; Andziak et al., 2006).

Although these may be isolated cases, they suggest oxidative stress alone may not predict lifespan. One possibility is that oxidative stress may impact changes in health and function associated with age, but not necessarily lifespan, suggesting value in the use of functional endpoint evaluation in aging studies (Lapointe & Hekimi, 2010). Also, it has been suggested that the paradoxical behavior of ROS may be attributed to environmental factors. For example, if mice are maintained in a stable environment (i.e. stable husbandry) then oxidative stress may not play a significant role in lifespan (Salmon, Richardson, & Perez, 2010). On the contrary, under normal conditions, ROS play an important role in signal transduction and cell proliferation, highlighting their paradoxical nature (Suzuki, Forman, & Sevanian, 1997). Many cell types release small oxidative bursts of ROS upon stimulation by growth factors, hormones and cytokines (i.e. interleukin 6 and tumor necrosis factor-α) and ROS are important in the initiation and function of various signaling cascades (Thannickal & Fanburg, 2000). For example, H_2O_2 can stimulate phosphorylation of serine/threonine and tyrosine residues or directly stimulate protein kinases (Valko et al., 2007). This is best exemplified through extracellular growth factors that rely on ROS as second messengers via the mitogen-activated protein kinase pathway (MAPK) pathway to ultimately activate the nuclear transcription factors involved in cell proliferation (Thannickal & Fanburg, 2000). There are many examples where ROS serve as signal mediators under normal conditions, and therefore maintaining a balanced intracellular redox environment is vital for physiological function. Further implication of free radicals in aging arises from the observation of reduced ROS-mediated and insulin-IGF-1 signaling in Ames and Snell dwarf mice

(Papaconstantinou, 2009). Global analysis of maximal longevity versus maximum mature mass for laboratory mice and rats suggests growth negatively impacts the lifespan of mammals (Rollo, 2002).

1.4 Growth and aging

The phosphoinositide 3-kinase pathway (PI3K) is among the key regulatory structures that modulate aging through various anabolic processes including protein synthesis, cell proliferation and growth (Spong & Bartke, 2010). The antagonism between growth (target of rapamycin signaling) and stress (FOXO) functions is mediated through PI3K, where protein kinase B (PKB) is the regulatory "switch" between the antagonistic pathways (Rollo, 2010). In mammals, PI3K plays a role in the development of numerous age-related pathologies including cardiac hypertrophy (Castello et al., 2011) and cancer (Tzivion & Hay, 2011). Therefore, recent efforts have involved targeting aspects of the PI3K regulatory network to attenuate the progression of age and disease. Furthermore, AMPK is a cellular energy sensor that regulates metabolic adjustments during periods of energy shortages (i.e. fasting or caloric restriction), and is strongly implicated in modern day aging frameworks. (Rollo, 2010; Canto & Auwerx, 2011). AMPK is among several kinases that can phosphorylate FOXO proteins that govern a suite of stress resistance functions (Rollo, 2010).

The insulin-IGF-1 system is linked with PI3K/Akt and plays a significant role in the lifespan of many organisms (i.e. *C.elegans* and *Drosophila melanogaster*), suggesting evolutionarily conserved signaling mechanisms (Carter, Ramsey, & Sonntag, 2002;

Kenyon, Chang, Gensch, Rudner, & Tabtiang, 1993; Tatar et al., 2001, Rollo, 2010). In vertebrates this axis consists of GH, insulin-like growth factors (IGFs), upstream hypothalamic molecules and downstream signaling molecules (Brown-Borg, Borg, Meliska, & Bartke, 1996; Rollo, Kajiura, Wylie, & D'Souza, 1999). The balance between hypothalamic regulatory factors such as growth hormone releasing hormone (GHRH) and somatostatin (SS) influence the release of GH (Bartke, 2009; Brown-Borg, 2009; Rollo et al., 1999). Subsequently, plasma GH stimulates the production and secretion of IGF-1 from the liver, in addition to local tissue-specific effects (Bartke, 2009; Rollo et al., 1999). The somatic axis influences of GH and IGF-1 include stimulating the growth of tissues and the metabolism of carbohydrate, lipid and protein (Brown-Borg, 2009). Therefore, alterations in GH and IGF-1 related pathways may disturb various metabolic processes and ultimately affect lifespan (Bartke, 2009).

Mouse models deficient in GH and IGF-1 (i.e. Snell and Ames dwarf mice) are commonly used models to investigate the role of IGF-1 and growth hormone in aging, because they lack the pituitary cells (mutation at *pituitary-1*) required for GH and thyroid-stimulating hormone secretion (Li et al., 1990). This generally translates into a greater than 50% increase in longevity in both Snell and Ames dwarf mice (Brown-Borg et al., 1996; Flurkey, Papaconstantinou, Miller, & Harrison, 2001; Selman et al., 2008). The trouble with these models is that several endocrine deficiencies may complicate our understanding of what exactly is manipulating longevity, in addition to phenotypic changes such as decreased body size, insulin sensitivity and increased antioxidant enzymes (Bartke, 2009). Further implicating the role of GH in aging are studies using

mouse models with a growth hormone receptor knockout (GHRKO), where males and females live 26 and 16% longer than wild-type litter mates (Coschigano et al., 2003). This is believed to be the result of high plasma GH and low plasma IGF-1 due to lack of GH binding in various tissues (Coschigano et al., 2003).

Part of the delayed-aging of these mice is attributable to enhanced antioxidant defenses correlating to reduced oxidative damage of proteins, lipids and mitochondrial DNA (Bartke et al., 2001; Romanick, Rakoczy, & Brown-Borg, 2004). In contrast, similar studies involving transgenic mice over-expressing growth hormone find significant reductions in lifespan and elevated free radical processes compared to wild-type (Rollo et al., 1996). These mice have shifted resource allocation towards excess lean growth, and exhibit signs of energy shortfalls based on changes in mass-specific food consumption and high carbohydrate preferences (Rollo et al., 1999). This exemplifies the bidirectional association of nutritional status and feeding behavior in the regulation of insulin-IGF-1 signaling. Also, the role of mammalian target of rapamycin (mTOR) is important as growth hormone functions (i.e. protein synthesis) are at least in part facilitated through this highly conserved signaling pathway that serves in the integration of growth, stress and nutrition signals (Zoncu, Efeyan, & Sabatini, 2011).

Ultimately, the insulin-IGF-1 system can modulate a number of processes including fat deposition, protein synthesis and most importantly, growth rates, which appear to correlate negatively with lifespan. Therefore, the regulation of growth at the whole-body and tissue-specific levels clearly exerts an influence on aging and longevity in mammals. However, subsequent changes at the subcellular level, including aspects of

mitochondrial function and metabolism are also implicated in aging. The decline of various physiological functions with age is associated with changes in metabolic parameters linked to oxidative stress and will be discussed below.

1.5 Aspects of energy metabolism, oxidative stress and aging

In mammals, the primary metabolic fuel sources include lipids, carbohydrates and proteins (Randle, 1995). The relative contribution of each substrate depends on the demand at any given time, and requires the coordination of several regulatory mechanisms to ensure an adequate energy supply. The challenge of this is highlighted by the variability of each fuel source in relation to energy density, amount stored, water solubility and the rate at which ATP is yielded (Weber, 2011).

Lipids possess characteristics that allow them to be better used during periods of prolonged physiological demands (i.e., fasting and/or low intensity exercise). Lipids are stored in large amounts because they are energy dense and therefore contain more joules per gram of energy compared to other substrates (Weber, 2011). Their low water solubility would otherwise pose a limiting factor with respect to transport, however this is achieved through the actions fatty acid binding proteins (FABPs) in the cytosol and albumin in plasma (van der Vusse, 2009). Although this allows for effective transport of lipids between tissues, it also highlights the possible limitations of lipid oxidation rates (Weber, 2011).

Carbohydrates by comparison are not only highly water soluble, but also exhibit high rates of ATP production, making them an ideal fuel source for anaerobic demands.

In addition, the oxidation of carbohydrates produces more ATP per mole of oxygen compared to proteins and lipids (Hochachka et al., 1991). However, because carbohydrates are low in energy density, their bodily stores are often small and can be rapidly depleted depending on the nature of its use (Weber, 2011).

Protein metabolism is important during periods of fasting (i.e. overnight sleep) or starvation, but generally it seems that animals tend to avoid protein oxidation for a number of reasons (McCue, 2010). For example, proteins provide essential amino acids and are used various membrane transport processes. Also, the oxidative breakdown of proteins produces the expected carbon dioxide and water (i.e. similar to lipid and carbohydrate oxidation), ammonia is a toxic byproduct as well (McCue, 2010). Therefore, most animals possess various physiological adaptations that minimize the use of proteins as a metabolic fuel source, except in times where carbohydrate and lipid stores become limited (McCue, 2010).

The manner in which these metabolic substrates are utilized is reflected in various metabolic parameters such as metabolic rate. Changes in metabolism over time can shed light on the role of energy balance in aging and disease. Total energy expenditure may play a role in aging and encompasses three key components of metabolism and that links back to Pearl's rate of living theory (Pearl, 1928). Basal metabolic rate (BMR) or resting metabolic rate (RMR) refers to the minimum energy required for the maintenance of body temperature and various physiological functions at rest and is usually measured following an overnight fast (post-absorptive state) (Frisard & Ravussin, 2006). In fact, RMR accounts for roughly 50-70% of daily energy expenditure (Tataranni, Larson, Snitker, &

Ravussin, 1995). Thermogenesis is the second component of total energy expenditure, and refers to the increase in metabolic rate typically associated with food intake or environmental factors such as temperature (Frisard & Ravussin, 2006). Thermogeic response to food intake account for roughly 10% of total energy expenditure (Ravussin & Swinburn, 1993). Physical activity is the third and most variable component that is comprised of exercise and non-exercise energy expenditure (also referred to as non-exercise activity thermogenesis) (Frisard & Ravussin, 2006; Levine, Eberhardt, & Jensen, 1999; Ravussin, Lillioja, Anderson, Christin, & Bogardus, 1986). In sedentary individuals, physical activity may account for approximately 20% of daily energy expenditure, and up to 50% in more active and/or athletically trained individuals (Frisard & Ravussin, 2006).

Since daily energy expenditure may play an important role in the aging process, it is important to consider the role of mitochondrial function as well. It would not be unreasonable to predict that the accumulation of oxidative damage with age is associated with metabolic rate (i.e. oxygen consumption). However, there is evidence to suggest that the oxidative capacity of mitochondria are compromised with age due to an accumulation of oxidative damage, allowing protons to leak at a higher rate than normal (Brand, Chien, Ainscow, Rolfe, & Porter, 1994). Findings from our laboratory and by others have shown that the enzymatic activity of mitochondrial electron transport chain complexes significantly declines with age in mice (Aksenov et al., 2010; 2011; Desai, Weindruch, Hart, & Feuers, 1996) and humans (Cooper, Mann, & Schapira, 1992). This is further supported by studies highlighting a ~50% decline in skeletal muscle ATP production in

elderly male humans (Petersen et al., 2003). Changes in membrane lipid composition with age may dysregulate the rate at which protons are leaked and may account for up to 30% of basal metabolic rate (Mookerjee, Divakaruni, Jastroch, & Brand, 2010). The leak of protons through the inner-mitochondrial membrane results in the uncoupling of substrate oxidative phosphorylation, thereby reducing the efficiency of ATP synthesis (Conley, Jubrias, Amara, & Marcinek, 2007). Although mitochondrial uncoupling can protect cells from ROS damage and has implications in lifespan (Speakman et al., 2004), energy deficiencies, particularly in older ages increases susceptibility to various diseases and lowers quality of life (Conley et al., 2007).

To better understand the link between mitochondrial function, oxidative stress and aging, researchers have characterized evolutionary adaptations in longer and shorter-lived species. Overall, inter-specific cross-species studies indicate that the link between metabolic rate and oxidative stress is positively correlated (Cutler, 1985; Ku, Brunk, & Sohal, 1993). However, in the same context, the link between oxidative stress and lifespan is not as well defined (Sohal & Weindruch, 1996; Speakman et al., 2004). The lack of clear-cut findings suggests that there may be other factors to consider when linking the role oxidative stress to longevity. This is reflected in the challenges of developing practical aging interventions that apply to the general population.

1.6 Dietary supplements: can they extend longevity?

The FRTA is not limited to damage caused by internal free radical sources (mitochondria, NOX, NOS) but also explains processes caused by external free radicals.

Likewise, attempts to extend lifespan are not limited to targeting endogenous antioxidant systems (SOD, catalase, etc.) but may include administration of free antioxidants via dietary supplementation. To the present day, antioxidant supplementation largely involved administration of simple dietary compounds containing one or a few ingredients. Successfully increasing lifespan through exogenous dietary antioxidants would not only principally support the FRTA, but would perhaps change aging interventions, as we know it. However, there have been many inconsistent and negative results leading to great skepticisms regarding the value of dietary supplementation to promote longevity. For example, resveratrol is highly regarded for its antioxidant properties (i.e. consumption of red wine), and although it successfully extended lifespan in *Caenorhabditis elegans* and *Drosophila melangoaster* (Bass, Weinkove, Houthoofd, Gems, & Partridge, 2007), success in mice was limited to improvements in several age-related pathologies (Baur et al., 2006). Furthermore, trials in mice involving dietary coenzyme Q10 (starting at 3.5 months of age) successfully elevated mitochondrial coenzyme Q10 content but did not alter antioxidant defenses, pro-oxidant generation or lifespan (Sohal et al., 2006). Vitamin C is a widely advocated and self-administered antioxidant that is believed to reduce oxidative stress and promote general health. Studies in mice using high life-long dietary doses of vitamin C show no improvement in mean or maximal lifespan, and may in fact reduce the capacity of endogenous antioxidant defenses (Selman et al., 2006). Also, life-long supplementation of vitamin E in mice increased median lifespan by 15%, however this increase was likely to be independent of any antioxidant effect, and more attributable to the anti-cancer properties of vitamin E (Banks, Speakman, & Selman, 2010). Although

there are numerous examples where dietary supplementation of antioxidants have either failed or extend lifespan in mice, there is considerable variability in doses, ingredients, method of administration and onset age of supplementation.

The age at which supplementation is initiated can be a critical factor in determining the success of a diet. Administration of antioxidant mixture containing beta carotene, alpha tocopherol, ascorbic acid, rutin, selenium and zinc at various ages (2, 9, 16 and 23 months of age), yielded significantly different results with regards to prolonging lifespan (Bezlepkin, Sirota, & Gaziev, 1996). Supplementation starting at 2 months of age prolonged the time of 100% mortality by 16%, and was most effective compared to any other age group (Bezlepkin et al., 1996). Furthermore, the administration of simple antioxidant combinations (i.e. vitamin E + glutathione) in middle-aged mice failed to alter disease incidence or extend longevity (Lipman et al., 1998). Ultimately, aging is complex and involves the gradual deterioration of multiple physiological pathways. Although certain vitamins, nutrients and dietary compounds may be effective in conserving function of individual mechanisms, single dietary compounds rarely prolong lifespan. Multi-component supplements that target several aspects of aging may show greater promise.

Our laboratory developed a complex dietary supplement (DSP) designed to offset five important mechanisms of aging: oxidative stress, inflammation, mitochondrial function, insulin resistance and cellular membrane integrity. We implement lifetime supplementation to maximize the synergistic interactions of the dietary components the potential to prolong physiological functions. Initial studies supplementing wild-type

C57BL/J (Nr) and an accelerated-aging transgenic mouse over-expressing growth hormone (Tg) extended mean longevity by 11 and 28%, respectively (Lemon, Boreham, & Rollo, 2005). Furthermore, the DSP afforded greater protection against radiation-induced DNA damage (i.e. 8-hydroxy-deoxyguanosine), and lowered levels of bone marrow chromosomal aberrations by 6-fold in supplemented Nr and Tg compared to untreated siblings (Lemon, Rollo, & Boreham, 2008). The DSP also maintained total daily locomotion in old supplemented mice compared to a >50% decline in untreated mice, boosted striatal neuropeptide Y, reduced brain protein carbonyls and reversed age-related declines in brain mitochondrial complex III activity (Aksenov et al., 2010).

Most recently we found that old supplemented mice performed significantly better in the Morris swim maze spatial learning task, and that brain weights increased even in young mice compared to controls (Aksenov et al., 2011). The benefits of our DSP extend beyond improving longevity, as performance in various functional measures were enhanced, and may translate into a better quality of life in elderly ages. Given that oxidative stress is one of the target mechanisms of our DSP, our results are in general support of the FRTA. However, several components of the supplement are included to ameliorate the four other mechanisms associated with aging mentioned, and therefore the synergistic interaction of these ingredients are thought to account for the exciting results we have obtained. Our work represents proof of principle that complex dietary supplements can be effective aging intervention strategies. In the following sections, research-involving aspects of metabolism and energy use in conjunction with our DSP will be presented and discussed.

1.7 Objectives and hypotheses

Aging is characterized by the progressive decline of function in various physiological regulatory pathways. Hence, attempts to slow the rate of aging should take on a multi-targeted approach since addressing just one part of a complex puzzle is unlikely to be successful. For this reason we developed a complex dietary supplement (DSP) designed to simultaneously target five key mechanisms of aging (oxidative stress, inflammation, mitochondrial function, insulin resistance and membrane integrity). At the same time, simply extending lifespan should not be the end goal if it comes at the expense of functional capacity in old age. Our DSP successfully extended the lifespan of Nr and Tg mice (Lemon et al., 2005). Beyond that, supplemented animals showed improvements in cognitive and motor function, decreased levels of oxidative damage biomarkers, and reversal of age-related declines in brain mitochondrial activity. This is likely to reflect aspects of metabolism and energy use in aging, and will be the focus of the work presented in the following sections.

Objectives:

1) Monitor age-related changes in metabolic parameters including basal metabolic rate, 24-hour patterns of oxygen consumption and physical activity in untreated and supplemented mice
2) Characterize markers of free radical background and antioxidant enzyme activity in heart, kidney and urine samples from untreated and supplemented mice across a broad age range

3) Assess whole-body glucose tolerance in older (≥ 12 months-old) untreated and supplemented Nr male mice

Hypotheses:

1) Supplemented mice will maintain youthful aspects of metabolism with age including basal metabolic rate and 24-hour oxygen consumption, compared to untreated controls
2) Supplemented mice will maintain youthful patterns of spontaneous physical activity with age compared to untreated controls
3) The DSP will lower markers of oxidative stress with age in kidney, heart and urine compared to untreated controls. This will be achieved by reducing free radical production and/or the up-regulation of antioxidant enzyme activity
4) A key mechanism of aging targeted by the supplement formulation was insulin sensitivity. If successful: Whole-body glucose tolerance will be improved in older supplemented Nr male mice compared to untreated controls

CHAPTER TWO: MATERIALS AND METHODS

2.1 Animals and dietary supplementation

Normal (Nr) and Transgenic (Tg) mice of C57BL/6J*SJL background were randomly bred. Briefly, the original stock was C57BL/6J male x SJL female hybrids, and the growth hormone (GH) transgenes (tandemly fused on one chromosome) are inherited in mendelian proportions. Therefore, equal numbers of Nr and Tg mice with similar genetic backgrounds are yielded by breeding Nr females to heterozygously Tg males. Tg were distinguishable after 28 days by their larger size. Tg carry a rat GH gene driven by a metallothionine promoter, and exhibit ~100-fold elevation in plasma GH. This results in doubled growth rate and adult mass compared to Nr mice, and a profound reduction in lifespan. All protocols were conducted in accordance with Canada Council on Animal Care guidelines.

At weaning, mice were randomly assigned to the untreated or supplemented diet groups for life. Untreated mice received only standard chow diet (Teklad 22/5, Madison, Wisconsin). The DSP is comprised of 30 ingredients **(Table 1)** designed to ameliorate five common mechanisms associated with aging. The supplemented group was given one dose per day of the ASP. Dosages were calculated based on daily recommended values for humans, and adjusted for the ten-fold higher mass-specific metabolic rate of mice. Small square pieces of bagel were marinated in a paste of the ASP ingredients, air-dried and dropped into cages housing supplemented mice (Lemon 2003; Aksenov 2011). Mice avidly retrieved and ate their entire bagel bit. Nr and Tg of a broad age range (2-31

months) from untreated and supplemented groups were randomly selected for all subsequent studies.

2.2 Spontaneous physical activity and indirect calorimetry

Basal metabolic rate (BMR) was measured for untreated and supplemented Nr male mice between 2-17 months-old following an 8 h fast. Respiratory gases were measured using an open flow-through respirometry system at room temperature (22-25°C). Animals were placed in a 600 mL glass resting chamber. Incurrent air entering the chamber was scrubbed of H_2O by drierite® (W.A. Hammond Drierite Company, Xenia, OH), and of CO_2 by soda lime and ascaraite (Fischer Scientific, Pittsburg, PA). Air entered the chamber with a constant flow rate of 600 ml/min using an MS5 mass-flow meter and pump (Sable Systems, Las Vegas, NV, USA). Excurrent air was scrubbed of H_2O by drierite and was sub-sampled at a rate of 80 ml/min by the O_2-CO_2 analyzer (Foxbox, Sable Systems, Las Vegas, NV, USA). Oxygen and carbon dioxide values were collected at a rate of 1 sample/5 seconds using a computer equipped with Expedata ® data acquisition software (Sable Systems, Las Vegas, NV, USA).

When measuring respiratory gases over a 24-h period (12:12 h light:dark cycle), a similar setup to the BMR study was used. However, non-fasted Nr male mice (2-27 months-old) were placed in a transparent acrylic chamber (L:19 cm; W:19 cm; H:11.5 cm) lined with woodchip bedding. The incurrent air flow rate was 800 ml/min, with a sub-sampling rate of 100 ml/min. Spontaneous activity levels (SpA) were recorded by placing the metabolic chamber on an analytical balance (Pinnacle Series Denver Instruments, Bohemia, NY, USA) to measure weight change triggered by movement

(Biesiadecki, Brand, Koch, & Britton, 1999). Oxygen, carbon dioxide, and SpA data were collected at a rate of 1 sample/5 sec to time-match the comparisons. To simplify the analysis of 24-h SpA, all samples where a weight change was registered by the animal was considered a spontaneous action regardless of the magnitude of weight change, while no registered weight change was considered resting or sleeping.

Feeding was monitored using pre-weighed food dishes placed inside the chamber and contained standard mouse chow pellets. Final food dish weights were recorded following 48-h incubation at ~42°C to remove moisture. Water bottles containing ~300 mL of water (by weight) were attached to a sipper tube secured to the outside wall of the metabolic chamber. Water consumption values were determined by the difference in weight of the water bottle after 24-h.

2.3 Gross morphological measurements of heart

Frozen heart samples were thawed for approximately 1-2 h and were gently blotted to remove fluid/blood remnants. The weights were then recorded in duplicate. Heart samples were dissected was completed using a surgical-grade #10 scalpel blade and surgical-grade micro scissors at 10X magnification. The atria were removed exposing the entrance to both ventricles. The left ventricle was dissected and weighed, followed by measures of wall thickness and intra-cavity diameter. All measurements were taken with the use of a standard scientific ruler or scientific calipers.

Fluctuations in body weight introduce variance in normalizing morphological features of the heart (and other organs). However, tibia length does not change after

maturity and is regarded as a better standard for normalizing index (Yin, Spurgeon, Rakusan, Weisfeldt, & Lakatta, 1982). Tibias were collected from euthanized Nr mice, and were removed just above the knee using surgical grade scissors. The tibia was dissected from the medial condyle to the media malleolus. Tibia length was measured using a standard scientific ruler and calipers.

2.4 Whole-tissue H_2O_2 background and catalase activity

Background H_2O_2 and catalase (CAT) activity was measured in heart and kidney tissue using an Amplex Red fluorescent probe (Invitrogen/Molecular Probes, Eugene, OR). A 10 mM Amplex Red stock solution was prepared in pure DMSO. The 10 U/mL (H_2O_2 background) and 100 U/mL (CAT activity) Horseradish Peroxidase (HRP) stocks were diluted in 0.25 M sodium phosphate reaction buffer, pH 7.4 and 0.5 M Tris-HCl, pH 7.5 reaction buffer, respectively. Excess Amplex Red and HRP stock solutions were aliquotted and stored at -20°C for up to 6 months. Aliquots were thawed immediately prior to use at room temperature. The 20 mM H_2O_2 and 1000 U/mL CAT solutions used to create standard curves were prepared fresh each day from the 3.0% H_2O_2 solution and 100 U/mL CAT stock provided. Heart and kidney samples were prepared according to the manufacturer's specifications.

Briefly, tissue homogenates were diluted 10-fold for H_2O_2 background and 20-fold for CAT activity in the final reaction mixtures. Samples were exposed to a 100 μM Amplex Red and 0.2 U/mL HRP working solution and incubated at room temperature for 30 minutes, (protected from light) to detect H_2O_2 background. When measuring CAT activity, samples were incubated with a 40 μM H_2O_2 working solution for 30 minutes at

room temperature, followed by incubation for 30 minutes at 37°C with 100 μM Amplex Red and 0.4 U/mL HRP working solution. Endpoint absorbance was measured at 560 nm using a SpectraMax Plus384® microplate reader (Molecular Devices Inc., Sunnyvale, CA., USA). One unit of catalase is the amount of enzyme that will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 10 mM hydrogen peroxide.

2.5 Oxidized:reduced glutathione ratio (GSSG:GSH)

Urine samples from older (≥12 months-old) Nr male and female mice in untreated and supplemented diet groups were collected following a 4-h fast. Samples were flash frozen in liquid nitrogen and stored at -80°C until processed for GSSG:GSH measurements. Samples were thawed to 4°C and de-proteinized, to eliminate potential interference with the fluorescence endpoint measurements. De-proteinization was completed by mixing urine samples with equal volumes of 5% sulfosalicylic acid (SSA) (Sigma-Aldrich #S2130) solution, followed by 10 minutes of incubation at 4°C. Samples then underwent centrifugation at 14,000 rotations per minute (RPM) for 10 minutes at 4°C. The supernatant was separated and processed within 2 h as outlined below.

GSH:GSSG was measured in deproteinized urine samples using a highly sensitive Amplite™ fluorometric glutathione GSH:GSSG Ratio assay kit (Cat# 10056, AAT Bioquest, Sunnyvale, CA., USA). Total GSH was determined using the Thiolite™ Green fluorometric GSH probe in accordance with manufacturer's recommendations. Briefly, 50 μl of 1 mM GSH and GSSG standards were serially diluted in duplicate over a 0 to 5 μM

and added to a solid a solid black 96-well microplate along with test samples. Subsequently, 50 µl of either GSH or GSSG reaction mixture (prepared from 400x Thiolite™ stock solution) was added to each well, followed by 60 minutes of incubation at room temperature. Fluoresence was measured using a Gemini™ XOS fluorescence microplate reader with excitation and emission spectra of 490 nm and 520 nm, respectively.

2.6 Intra-peritoneal glucose tolerance test (IPGTT)

Randomly selected older (≥12 months old) Nr male mice from untreated and supplemented groups were fasted for 8 h, with only water available *ad libitum*. Mice were housed individually in cages lined with woodchip bedding (#7090 Sani-Chips,® Harlan Laboratories, Mississauga, ON) and contained an enrichment object. Following the baseline blood glucose measurement (ACCU-CHEK Compact Plus glucometer, Hoffmann-La Roche Ltd.), mice were given an intra-peritoneal injection of 1.0 g/kg D-glucose (Sigma-Aldrich #G-7528) solution prepared (0.25 g/mL) and incubated overnight at room temperature to reach equilibrium between the α- and β-glucose isoforms. Blood glucose measurements were taken from a small cut on the tail tip at 15, 30, 60, 90 and 120 minutes post-injection. Values are presented as mean ± SEM (mmol/L).

2.7 Statistics

All statistical analyses were performed using Statistica® 8.0 and Graphpad Prism 5.0 software. Age-related trends of dependent variables were characterized with linear

regression and the impacts of diet were assessed using analysis of covariance (ANCOVA), where age or time (e.g., hourly breakdown of calorimetry data) was the covariate. Body mass did not significantly vary between diets within Nr and Tg groups, and was removed from subsequent analyses. Rather, free radical and antioxidant enzyme measures were adjusted on the basis of tissue weight (i.e. mass-specific), therefore, cross comparisons between Nr and Tg did not directly involve body weight. Differences in age-related trends were distinguished using general linear model homogeneity of slopes analysis, and will be referred to as separate-slope analysis. However, age effects were also considered in these analyses, as data sets were subdivided into young (≤ 12 months-old) and old (> 12 months-old). A 12 month dividing point was used because it best captured middle-old aged Nr and old Tg, best suiting the natural division of age ranges of animals used in this study. In cases where age, time or diet effects were not significant, mean group values of dependent variables were compared using student *t*-tests and one- or two-way ANOVAs for repeated measures where appropriate.

For whole-body glucose tolerance, area under the curve (AUC) was determined using the trapezoid rule using Graphpad Prism 5.0 software. Mean AUC values for each diet group represent an average of individual AUC values of each animal. For analyses where in which significant effects were detected, Student-Newman-Keuls (ANCOVA), Bonferroni (two-way ANOVA) and Tukey HSD (one-way ANOVA) *post hoc* tests were applied used. All data are presented as means ± standard error of the mean (SEM). Statistical significance was accepted with a criterion of $p < 0.05$.

Table 1. List of DSP ingredients

Ingredient	Mouse Dose (mg/day/100 mice)*
Vitamin B1	30.49
Vitamin B3 (Niacin)	30.49
Vitamin B6	60.98
Vitamin B12	0.18
Vitamin C	350.61
Vitamin D	0.02
Acetyl L-Carnitine	146.45
Alpha-Lipoic Acid	182.93
Acetylsalicylic acid	132.11
Beta Carotene	21.95
Bioflavonoids	792.68
Chromium Picolinate	0.3
Folic Acid	0.61
Garlic	3.81
Ginger Root Extract	600.37
GinkoBiloba	18.29
Ginseng	631.1
Green Tea Extract	487.8
L-Glutathione	30.49
Magnesium	45.73
Manganese	19.05
Melatonin	0.73
N-Acetyl Cysteine	304.88
Potassium	18.11
Rutin	304.88
Selenium	0.05
Vitamin E	326.83
Cod Liver Oil (Omega 3)	1219.51
CoEnzyme Q10	60.98
Flax Seed Oil	1219.51

CHAPTER THREE: RESULTS

3.1 Spontaneous physical activity (SpA)

Twenty-four hour spontaneous activity (SpA) with respect to age is shown in **Fig. 1** for untreated and supplemented male mice. Linear regressions of age-related trends (**Fig. 1A**) highlighted a significant decline in spontaneous locomotion of old untreated males (-16%, **p = 0.014**) by 24 months of age. The modest 10% decline in SpA of supplemented males was not resolved (p = 0.23). Although regression slopes (untreated: *b* = -118.1; supplemented: *b* = -63.37) did not significantly vary (p = 0.40), supplemented males generally maintained higher 24-hour SpA causing the age regression to be significantly (**p = 0.028**) elevated compared to untreated males. When the effect of age is omitted, a comparison of mean 24-hour SpA underlines a significant (**p = 0.032, Fig. 2A**) dietary effect on SpA (untreated: 12349 ± 393; supplemented: 13503 ± 429 actions per day). To determine the effect of time, age-related trends of total 24-hour SpA values were separated into 12-hour light **(Fig. 1A)** and dark **(Fig. 1B)** periods. The DSP abated age-associated declines in SpA during both photoperiods. During the photophase, the regression slope of untreated males was significantly non-zero **(***b* = -71.69**, p = 0.032**), exhibiting a 25% decline in spontaneous locomotion at ~24 months. No significant changes in photophase SpA were resolved for supplemented males (*b* = -21.67, p = 0.50), however, scotophase SpA were higher in both diet groups compared with the photophase. Furthermore, SpA significantly declined with age in untreated mice (-15%, *b* = -50.58; **p = 0.0064**), while no significant age-related changes were resolved for supplemented mice (*b* = -34.36**,** p = 0.29). **Fig. 2B** illustrates the overall impact of diet and photoperiod on

mean SpA, when age is excluded from the analysis. The 27% decline observed in mean photophase SpA compared with the scotophase, indicates a highly significant **(p = 0.0001)** effect of time of day in the untreated group (-1390 ± 305 actions). Supplemented mice showed only a marginal decline (-10%, -599 ± 365 actions; p = 0.06) in mean photophase SpA compared to the dark. Within the photophase mean SpA of supplemented males was 19% (+980 ± 365 actions, **p = 0.0075)** higher compared to untreated mice, however, statistical significance was not achieved when comparing mean scotophase SpA (untreated: 6459 ± 157 vs. supplemented: 6649 ± 263; p = 0.27).

The effects of diet on age-related changes and mean number of hours spent resting in a 24-hour period are shown in **Figs. 3A** and **3B** respectively. Age-related regressions of both untreated (b = 0.18) and supplemented (b = 0.11) males indicate increases in time spent resting. At ~24 months, untreated mice showed a linear 60% increase (**p = 0.0094**) in number of hours resting compared with young animals. By contrast, the ~50% age-related increase in resting observed in supplemented males was not statistically resolved (p = 0.17). Although age slopes did not significantly vary between diets, the elevation of the regression for untreated males was significant **(p = 0.024)**. Furthermore, mean number of hours resting per day was ~25% higher in untreated compared to the supplemented group (6.9 ± 0.56 and 5.2 ± 0.65 hours/day respectively; **p = 0.031**).

Food and water consumption was monitored for all animals during 24-hour respirometry trials. Overall, mean gram per day food consumption was nearly identical (untreated: 5.62g vs. supplemented 5.76 g per day; p = 0.96) in animals from both diets. A closer look at age shows that young mice consumed ~7 g of food per day; however, as

mice aged food consumption dropped approximately 28%, to ~5g per day. Although untreated and supplemented males showed very similar age-related trends in food consumption, only the regression of untreated mice was resolved as significantly non-zero (Untreated: slope (*b)* = -0.21, **p = 0.027**; Supplemented: *b* = -0.13, p = 0.12). Finally, separate-slope analysis confirmed that neither slopes (p = 0.46) or intercepts (p = 0.51) differed significantly between regression lines.

Changes in 24-hour water consumption (ml/day) are reported in **Fig. 4**. Interestingly, while the ~10 ml of water per day consumed by young mice in both diet groups **(Fig. 4A)** remained unchanged (p = 0.92) in old supplemented mice, untreated males progressively consumed more water with age, culminating in a ~50% increase by 24 months, compared to age-matched supplemented males. Regression slopes varied significantly (p = 0.049, separate-slope ANCOVA). When the effect of age is omitted (**Fig. 4B**), the effect of diet remained significant (p = 0.18), with untreated males (12.3 ± ml/day) consuming more water than supplemented males (9.8 ml/day)

3.2 Indirect calorimetry

Basal metabolic rate measurements were taken from fasted Nr male mice in untreated and supplemented diet groups. VO_2 reflects the volume of oxygen consumed over a particular fixed time period, and is expressed as ml/kg/min. A general linear model (separate slopes) indicates opposite age-related trends between diets **(p = 0.018, Fig. 5A)** and resolved a significant (**p = 0.0036**) effect of diet and a diet x age interaction (**p = 0.018**) in relation to VO_2. Oxygen consumption progressively declined with age in

untreated (*b* = -0.59) mice, however the relationship did not achieve statistical significance (p = 0.13). Young supplemented males consumed 25% less oxygen compared with age-matched untreated males, and overall showed a ~19% increase in oxygen consumption with age (*b* = 0.52, **p = 0.048**). The greatest differences are observed in young mice as age regressions converge by ~12 months. Mean VO_2 (excluding age) in untreated males was ~15% higher (**p = 0.045, Fig. 5B**) than supplemented males.

The respiratory quotient (RQ) is a dimensionless value calculated as the ratio between volume of carbon dioxide produced (VCO_2) per unit of oxygen consumed (VO_2) ($RQ = VCO_2/VO_2$), and provides an estimate of relative changes in the transition of fat and carbohydrate oxidation (RQ = 0.7 and 1.0 respectively). Similar to VO_2, RQ in untreated mice was found to significantly (**p = 0.021, Fig. 6A**) decline toward increased fat oxidation, while supplemented males showed non-significant (p=0.16) increases in relative proportion of CHO oxidation with age. Notably, a general linear model (separate slopes) resolved a significant difference between slopes indicating a diet x age interaction effect (untreated: *b* = -0.0033; supplemented: *b* = 0.0025; **p = 0.010**). No differences (p = 0.35, **Fig. 6B**) were resolved between mean RQ values of each diet group (untreated: 0.75 ± 0.0067 and supplemented: 0.76 ± 0.0060, p = 0.35) when age was excluded from the analysis.

Non-fasted resting VO_2 and resting respiratory exchange ratio (RER) data were extracted from the first hour in each of the 24-hour calorimetry trials. Untreated mice exhibited a highly significant **(-40%; p = 0.0041, Fig. 7A)** decline in resting VO_2 with

age, but this remained unchanged in the supplemented group ($p = 0.56$). Separate-slope analysis confirms significantly **($p = 0.027$)** different age-related trends in resting VO_2 between diets. The greatest differences were observed between the youngest mice (3-6 months-old), with the untreated group consuming ~20% more oxygen at rest. Overall, mean resting VO_2 did not significantly ($p = 0.29$, **Fig. 7B**) differ between diets when the effects of age were excluded.

Similar to non-fasted resting VO_2, untreated mice showed a significant **($p = 0.013$, Fig. 8A)** age-related decline in resting RER, whereas no significant changes were observed in the supplemented group. Although age slopes did not significantly vary between diets ($p = 0.14$), the supplemented regression was significantly elevated compared to untreated mice **($p = 0.0091$)**, resulting in a higher overall resting RER **($p = 0.022$, Fig. 8B)**.

As expected, scotophase VO_2 was ~19% **($p = 0.038$)** higher compared to light, regardless of diet. Consistent with our findings of non-fasted resting VO_2, untreated mice showed a 40% **($p = 0.041$, Fig. 9A)** drop in VO_2 with age, while supplemented mice remain virtually unchanged ($p = 0.88$). Although the difference between the slopes was only marginally significant ($p = 0.09$), mean VO_2 of young (3-5 months-old) untreated mice was ~30% higher than age-matched supplemented mice. Despite observing nearly identical trends in 24-hour VO_2 during the scotophase, the untreated regression was not statistically resolved ($b = -0.84$, $p = 0.14$, **Fig. 9B**).

An hourly breakdown of VO_2 patterns over 24 hours revealed a highly significant effect of diet (**$p < 0.0001$**, **Fig. 10A**), with supplemented males consistently respiring less

oxygen during both the scoto- and photophases. Animals were divided into young (≤ 12 months-old) and old (> 12 months-old) to better understand changes in hourly VO_2 with age. Surprisingly, the highly significant effect of diet on hourly VO_2 patterns was conserved in young mice (**p < 0.0001**, **Fig. 10B**), but was only marginal in the old group (p = 0.068, **Fig. 10C**). This relationship held true when comparing mean hourly VO_2 values (**Fig. 10D**) using a two-way ANOVA to assess the impacts of diet (**p < 0.0001**) and age (**p < 0.0001**). Specifically, the DSP lowered mean hourly VO_2 in young supplemented by ~19% compared to untreated counterparts, while only a non-significant 8% difference was seen in old males. In spite of this, we observe a highly significant reduction (~13%) in mean hourly VO_2 in supplemented males when age groups were combined.

We find a positive correlation in both diet groups when mean hourly VO_2 values were plotted against hourly SpA (**Fig. 11**). Though regression slopes of untreated and supplemented mice were nearly identical (*b* = 0.029 and 0.028 respectively) and significantly non-zero (**p = 0.0018** and **0.0022** respectively), the untreated regression was significantly (**p < 0.0001**) elevated above that of supplemented mice. This indicates untreated mice consume more oxygen to achieve comparable levels of activity over time.

3.3 Whole-tissue H_2O_2 background and catalase activity

3.3.1 Heart tissue:

Prior to measurements of catalase activity, heart samples from Nr and Tg mice were quickly measured for gross morphological features. In particular, we were interested

in left ventricle wall thickness (mm) as this is considered a primary indicator of cardiac hypertrophy. We find that in Nr supplemented males, mean left ventricular wall thickness was significantly lower (**p = 0.017, Table 2**) compared with untreated Nr, after being corrected using tibia length (untreated: 0.12 ± 0.012; supplemented: 0.08 ± 0.008). In addition, left ventricle wall thickness was also significantly reduced in supplemented Tg (**p = 0.028**) compared with untreated Tg (untreated: 0.065 ± 0.008; supplemented: 0.047 ± 0.004). However, for Tg, body weight was used as the correction factor as tibias from these animals were unavailable. A summary of mean values is shown in Table 2 for both Nr and Tg.

We measured free radical H_2O_2 background levels in heart samples collected from Nr and Tg male mice across a broad age range (3-31 months-old and 4-23 months-old respectively) to closely examine changes with time. The DSP did not significantly alter age-related trends in either genotype (**Fig. 12**). This was confirmed by a comparison of mean background H_2O_2 values, where no statistically significant differences were resolved (Nr: p = 0.54; Tg: p = 0.74, **Fig. 13A**) mice, although free radical background was found to be significantly higher in the Tg group (**p = 0.021, Fig. 13B**).

Almost identical age-related trends in catalase activity (U/ml) were observed in both Nr (**Fig. 14A**) and Tg (**Fig. 14B**) groups. Untreated mice (both Nr and Tg) maintained catalase activity across all ages (p = 0.19 and 0.18 respectively), but young supplemented males showed more than double (Nr: +55% and Tg: +50%) the catalase activity of age-matched untreated counterparts, with gradual declines by old age (Nr: **p = 0.030 and Tg: 0.035**). Separate-slope analysis resolved significant differences between

age regressions (**p = 0.039** for both Nr and Tg). Mean catalase activity is ~33% higher in supplemented males from both Nr (**p = 0.016, Fig. 15A**) and Tg (**p = 0.038**) groups compared with untreated counterparts. Overall, the catalase activity was ~30% (**p = 0.0018, Fig. 15B**) lower in Tg males compared to Nr, regardless of age group.

3.3.2 Kidney tissue

Unlike the observations reported in heart samples, H_2O_2 background was differentially impacted by our DSP in kidney. In Nr males the slopes of age regressions of both untreated (-2%, *b* = -0.19) and supplemented (+2%, *b* = 0.35) male mice were individually found to be significant (**p = 0.025,** and **0.045** respectively, **Fig. 16A**). Taken together, separate-slope analysis resolved a very significant difference between diet (**p = 0.0041**). In transgenic males, H_2O_2 showed a general non-significant decline with age regardless of diet group (p = 0.42, **Fig. 16B**) between slopes of untreated and supplemented males. Despite the opposite age-related trends between dietary treatments in Nr male mice, mean H_2O_2 did not differ (p = 0.87, **Fig. 17A**), and this was also the case for Tg males (p = 0.60). However, a comparison between Nr and Tg males (pooled diet groups) suggests mean H_2O_2 background is significantly higher in kidneys from Nr males (**p = 0.023, Fig. 17B**).

In contrast to the DSP's augmentation of catalase activity in heart from both Nr and Tg mice, no comparable elevation was seen in kidney. Separate slope indicated changes in catalase activity with respect to age were very similar between diets in both Nr (p = 0.43, not shown) and Tg (p = 0.32) male mice. Overall, mean catalase activity

remained unchanged in both Nr (untreated: 2.7 U/ml and supplemented: 2.3 U/ml; p = 0.29, **Fig. 18A**) and Tg (untreated: 3.4 U/ml and supplemented: 3.5 U/ml; p = 0.68) males. Finally, a highly significant genotypic difference (pooled diet groups) was found, with mean catalase activity in kidneys being ~36% higher in Tg males ($p < 0.0001$, **Fig. 18B**), compared with Nr counterparts.

3.4 Glutathione Ratio (GSH:GSSG)

The ratio between reduced versus oxidized glutathione pools in urine reflects general whole-body oxidative stress. We measured this ratio in urine samples collected from old (> 12 month-old) Nr male mice on either a control or supplemented diet. Differences between mean GSH:GSSG ratios (untreated: 0.65 and supplemented: 0.93) indicated a 43% (**p = 0.0004, Fig. 19**) increase in reduced glutathione in urine samples from old supplemented mice, indicative of remarkably lower oxidative stress.

3.5 Whole-body glucose tolerance

Whole-body glucose tolerance was measured in old (> 12 month-old) untreated and supplemented Nr male mice. The DSP significantly improved blood glucose (mmol/L) clearance rates as values peaked by the 15 minute time point in old supplemented males compared to 30 minutes in untreated counterparts. Furthermore, by 60 minutes supplemented males had significantly lower blood glucose (untreated: 13.3 mmol/L and supplemented: 10.9 mmol/L; **p = 0.0075, Fig. 20**), although total AUC varied only marginally (779.4 vs. 676.1 mmol/L per 120 min, p = 0.056).

Figure 1. Age-related trends of 24-hour spontaneous activity (SpA) in normal untreated and supplemented male mice.

Age-related trends of SpA in normal untreated (n = 10; 2-23 months-old) and supplemented (n = 10; 2-27 months-old) male mice during (A) combined photo- and scoot-phase (24-hours), (B.) photophase only (12-hours) and (C.) scotophase only (12-hours). Overall, only untreated mice exhibited significant age-related losses in SpA (**p = 0.014**). The effect of photoperiod on SpA with respect to age was highly significant (**p < 0.0001**), with ~17% higher activity during the dark.

Linear Regressions:

(A.) Untreated: $y = 14137 – 118.1*x$; $r^2 = 0.55$; **p = 0.014**

Where y = number of spontaneous actions and x = age (months)

Supplemented: $y = 14488 – 63.37*x$; $r^2 = 0.18$; p = 0.23

Difference in elevation of slopes: **p = 0.028**

Effect of diet: **p = 0.028**

Effect of age: **p = 0.012**

Diet x age interaction: **p = 0.028**

(B.) Untreated: $y = 6154 – 71.69*x$; $r^2 = 0.46$; p = **0.032**

Supplemented: $y = 6386 – 21.67*x$; $r^2 = 0.059$; p = 0.49

Difference in elevation of slopes: **p = 0.0086**

Effect of diet: **p = 0.009**

Effect of age: n.s.

(C.) Untreated: $y = 7225 – 50.58*x$; $r^2 = 0.63$; **p = 0.0064**

Supplemented: $y = 7183 – 34.36$; $r^2 = 0.14$; $p = 0.29$

Effect of diet: n.s.

Effect of age: **p = 0.027**

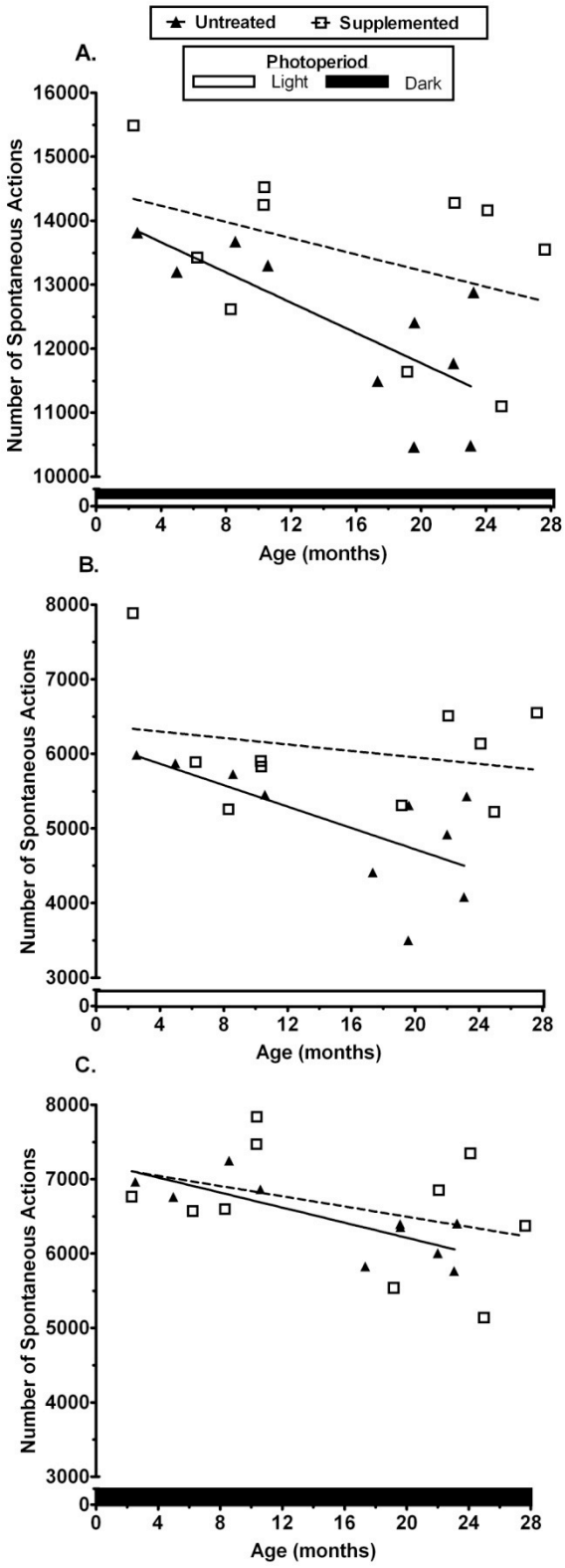

Untreated
Supplemented
A.
Photoperiod
Light
Dark
Number of Spontaneous Actions
16000
15000
14000
13000
12000
11000
10000
0
0
4
8
12
16
20
24
28
Age (months)
B.
Number of Spontaneous Actions
8000
7000
6000
5000
4000
3000
0
0
4
8
12
16
20
24
28
Age (months)
C.
Number of Spontaneous Actions
8000
7000
6000
5000
4000
3000
0
0
4
8
12
16
20
24
28
Age (months)

Figure 2. Effect of diet on mean 24-hour spontaneous activity (SpA) in normal untreated and supplemented male mice.

Changes in mean SpA in untreated (n = 10) and supplemented (n = 10) male mice over (A.) combined photophases and (B.) individual photo- and scotophase. Overall, supplemented males were 9% more active (**p < 0.0001**). Data are presented as means ± SEM. Significance is indicated by overhead bars: ***** ($p < 0.05$), ****** ($p < 0.01$), ******* ($p < 0.001$)

Summary of means:

(A.) Untreated: 5559 ± 210; n = 20

Supplemented: 6554 ± 150; n = 20

Difference between means = 995 ± 258, **p = 0.032**

Summary of means:

(B.) **Light**

Untreated: 5069 ± 262; n = 10

Supplemented: 6049 ± 253; n = 10

Difference between means: 980 ± 364, **p = 0.0075**

Dark

Untreated: 6459 ± 158; n = 10

Supplemented: 6649 ± 263; n = 10

Difference between means: 190 ± 306, p = n.s.

Cross-comparison (t-test)

Untreated: light vs. dark, **p = 0.0001**

Supplemented: light vs. dark, p = 0.056

Effect of diet: **p < 0.0001**

Effect of photoperiod: **p = 0.0056**

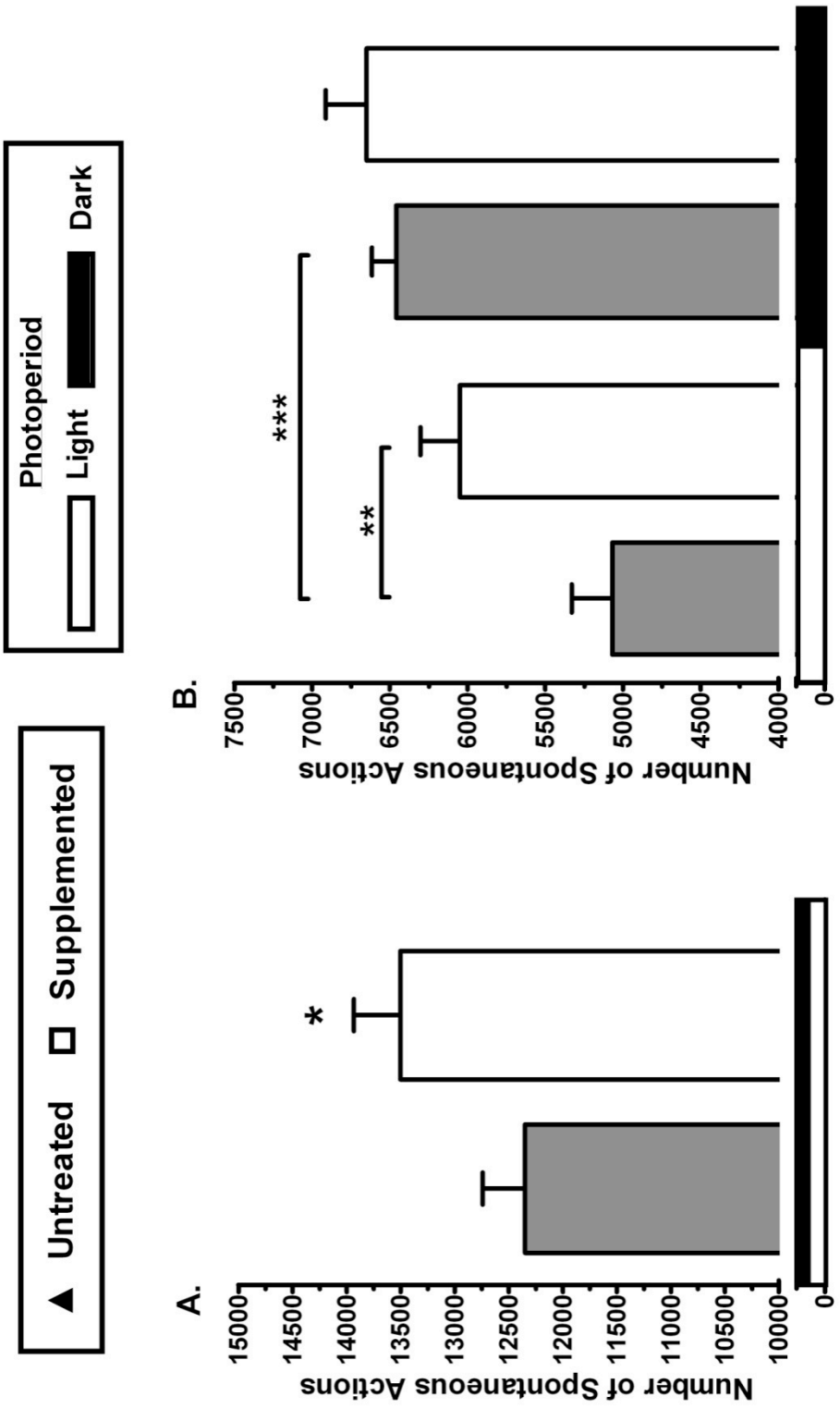

Untreated
Supplemented
Photoperiod
Light
Dark
A.
Number of Spontaneous Actions
15000
14500
14000
13500
13000
12500
12000
11500
11000
10500
10000
0
*
B.
Number of Spontaneous Actions
7500
7000
6500
6000
5500
5000
4500
4000
0
**
***

Figure 3. Age-related trends of hours spent resting in a 24-hour period between untreated and supplemented mice.

The impact of diet on (A.) time spent resting (hours per day) with respect to age and (B.) mean hours resting per day between untreated (n = 10; 2-23 months-old) and supplemented (n = 10; 2-27 months-old) male mice. Data are presented as hours spent resting per day versus age (months) per animal (A.) and mean ± SEM of hours spent resting (B.) between untreated and supplemented diets. Significance: * ($p < 0.05$) versus untreated males.

Linear regressions:

(A.) Untreated: $y = 4.25 – 0.175*x$; $r^2 = 0.59$; **p = 0.0094**

Where x = age (months) and y = number of hours resting per day

Supplemented: $y = 3.54 – 0.11*x$; $r^2 = 0.22$; $p = 0.2260$

Difference between slopes: $p = 0.46$

Difference in elevation of slopes: **p = 0.024**

Summary of means:

(B.) Untreated: 6.897 ± 0.5609; n=10

Supplemented: 5.194 ± 0.6472; n=10

Difference between means: 1.703 ± 0.87, **p = 0.031**

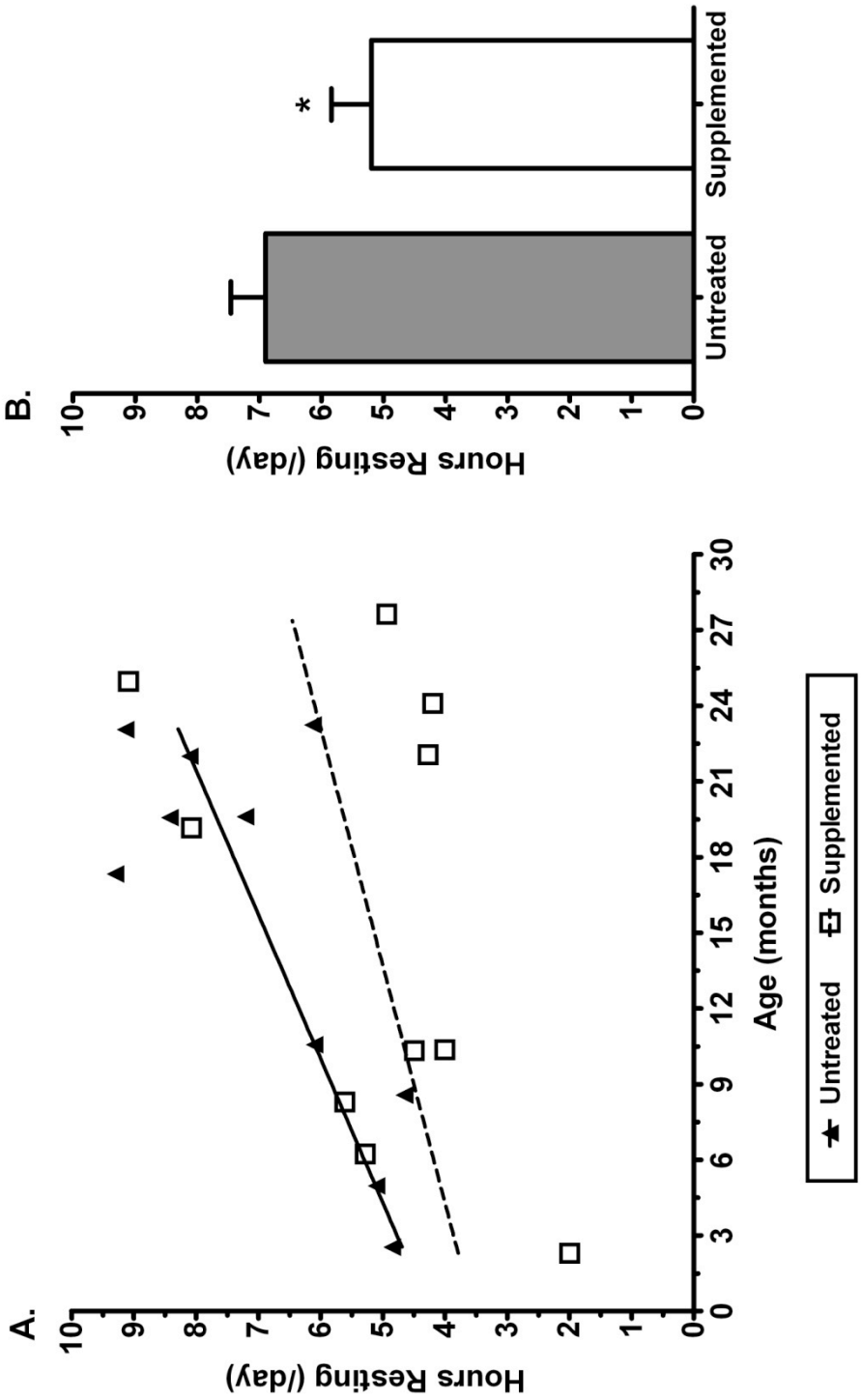

A.
Hours Resting (/day)
Age (months)
Untreated
Supplemented
B.
Hours Resting (/day)
Untreated
Supplemented
*

Figure 4. Age-related trends of 24-hour water consumption between untreated and supplemented mice.

Differences in 24-hour water consumption (ml/day) between untreated (n = 19; 2-25 months-old) and supplemented (n = 16; 2-30 months-old) male mice, (A.) with respect to age between and (B.) mean values. Data are presented as 24-hour water consumption values per animal versus age (A.) and mean ± SEM water consumption by diet group (B.). Significance: * ($p < 0.05$) versus untreated males.

Linear regressions:

(A.) Untreated: $y = 9.66 + 0.22*x$; $r^2 = 0.29$; **p = 0.018**

Where x = age (months) and y = H_2O consumption (ml/day)

Supplemented: $y = 9.96 – 0.0076*x$; $r^2 = 0.0007$; $p = 0.92$

Difference between slopes: **p = 0.017**

Summary of means:

(B.) Untreated: 12.31 ± 0.71; n=19

Supplemented: 9.850 ± 0.65; n=16

Difference between means: 2.458 ± 0.98; **p = 0.018**

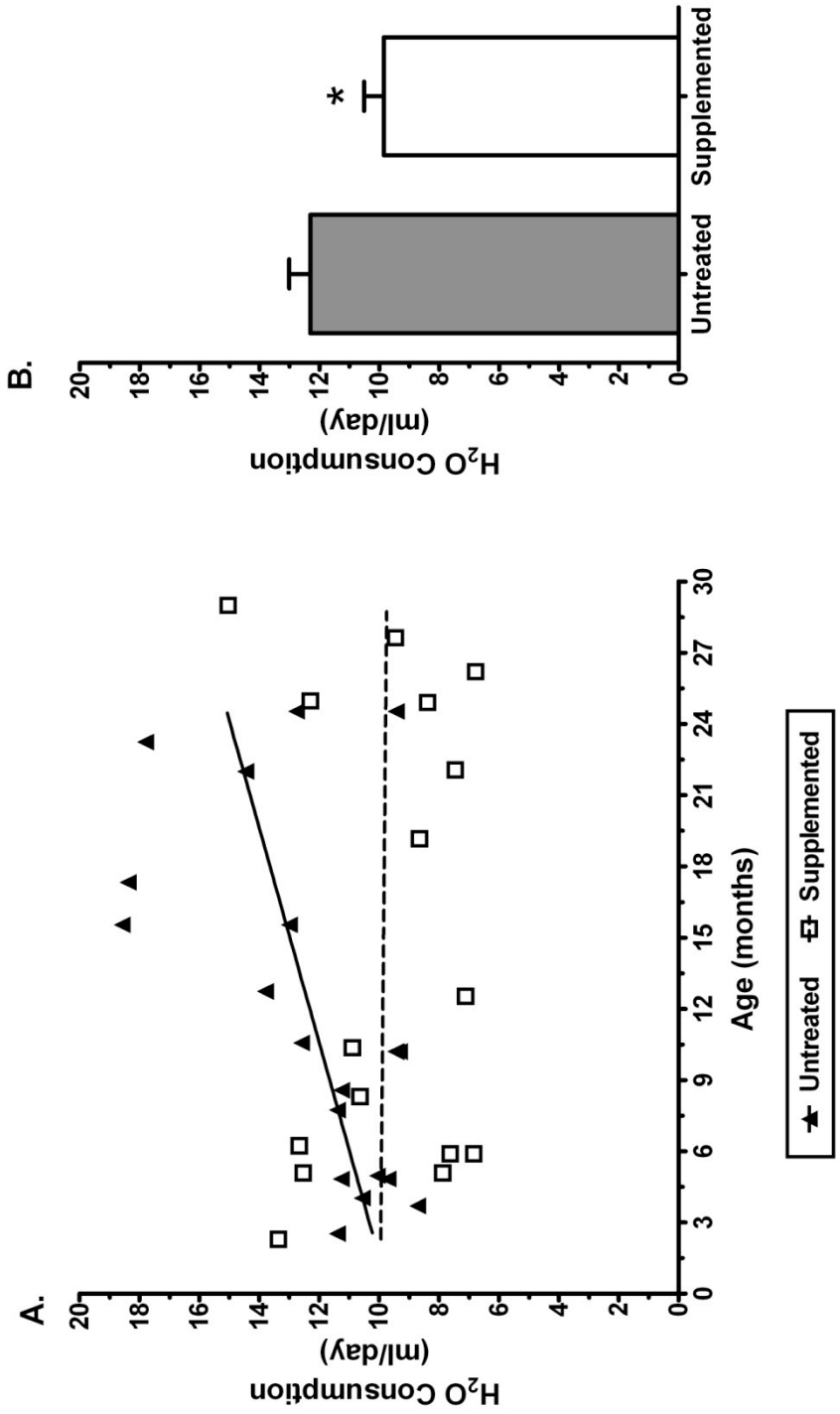

A.
H2O Consumption (ml/day)
Age (months)
Untreated
Supplemented
B.
H2O Consumption (ml/day)
Untreated
Supplemented
*

Figure 5. Basal metabolic rate measurements: Comparison of age-related trends and mean resting VO_2 of normal untreated and supplemented male mice.

Basal metabolic rate measurements of VO_2 (ml/kg/min) (A.) with respect to age and (B.) diet group in normal untreated (n = 9; 4-13 months-old) and supplemented (n = 11; 4-16 months-old) male mice in a fasted state. Overall, only the supplemented regression was significant, but slopes differed significantly **(p = 0.018)**. Mean VO_2 was significantly higher in untreated mice (**p = 0.045**). Data reported are means ± SEM. Significance: * ($p < 0.05$) versus untreated males.

Linear regressions:

Untreated: $y = 38.46 – 0.59*x$; $r^2 = 0.29$; $p = 0.13$

Where x = age (months) and y = VO_2 (ml/kg/min)

Supplemented: $y = 25.21 + 0.52*x$; $r^2 = 0.37$; **p = 0.048**

Difference between slopes: **p = 0.018**

Effect of diet: **p = 0.049**

Summary of means:

Untreated: 33.53 ± 1.707; n = 9

Supplemented: 29.54 ± 0.9309; n = 11

Difference between means: 3.989 ± 1.850. **p = 0.045**

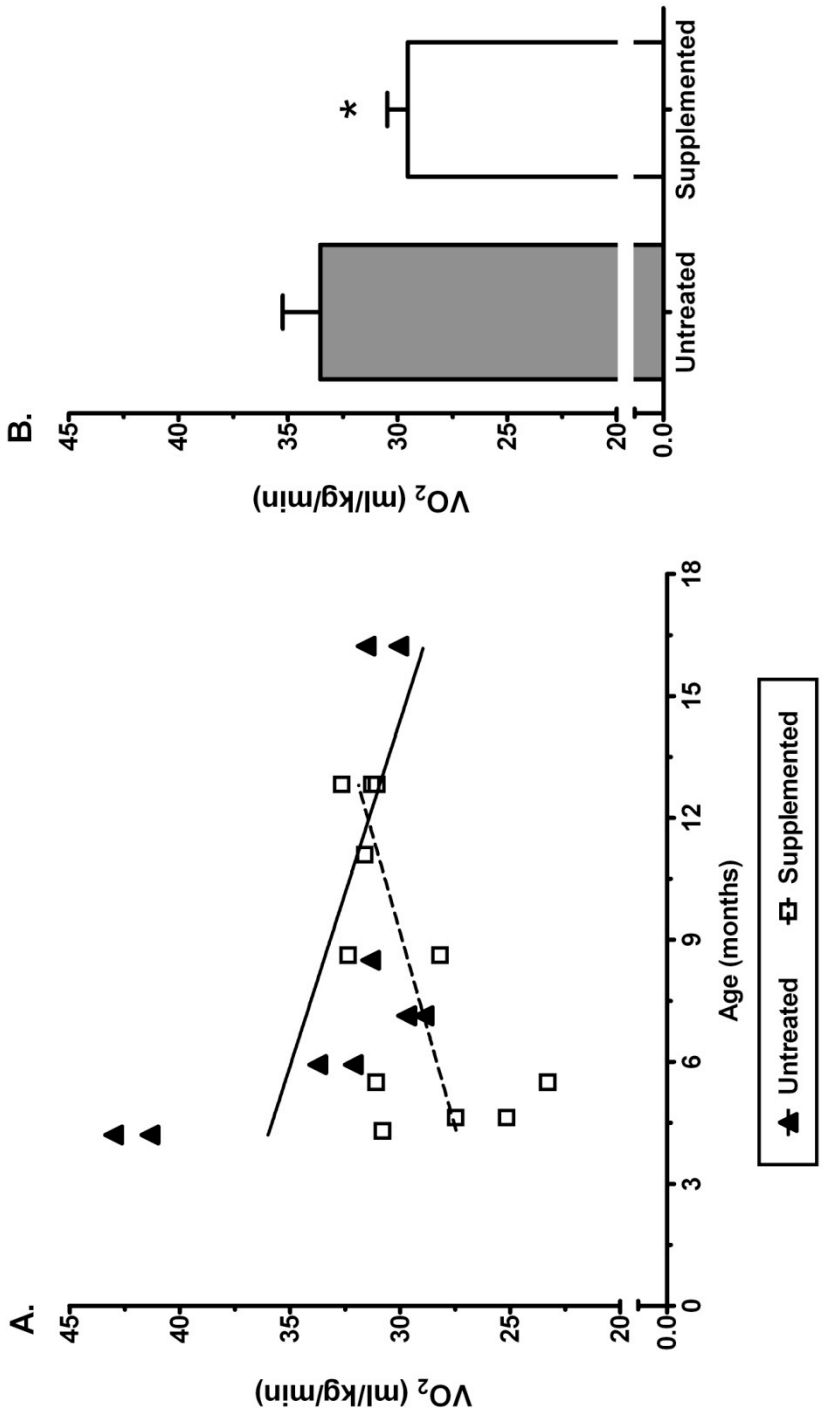

A.
B.
VO2 (ml/kg/min)
Age (months)
Untreated
Supplemented
*
0.0
0
3
6
9
12
15
18
20
25
30
35
40
45

Figure 6. Basal metabolic rate measurements: Comparison of age-related trends and mean resting respiratory quotient (RQ) of untreated and supplemented mice in a fasted state.

Basal metabolic rate measurements of RQ (A) with respect to age and (B) diet group in normal untreated (n = 9; 4-13 months-old) and supplemented (n = 11; 4-16 months-old) male mice in a fasted state. Age-related trends in fasting RQ at rest. Untreated mice showed a significant **(p = 0.021)** age-related decrease in fasting RQ at rest, while no significant differences were resolved for the supplemented group. Overall, the slopes of age-related trends differed significantly **(p = 0.010)** between diets but mean values did not. Data reported are means ± SEM.

Linear regressions:

(A.) Untreated: $y = 0.77 - 0.0032*x$; $r^2 = 0.55$; **p = 0.021**

Where x = age (months) and y = respiratory quotient

Supplemented: $y = 0.74 + 0.0025*x$; $r^2 = 0.20$; p = 0.16

Difference between slopes: **p = 0.010**

Summary of means:

(B.) Untreated: 0.7515 ± 0.0067; n = 9

Supplemented: 0.7601 ± 0.0060; n = 11

Difference between means: -0.0086 ± 0.0090, p = n.s.

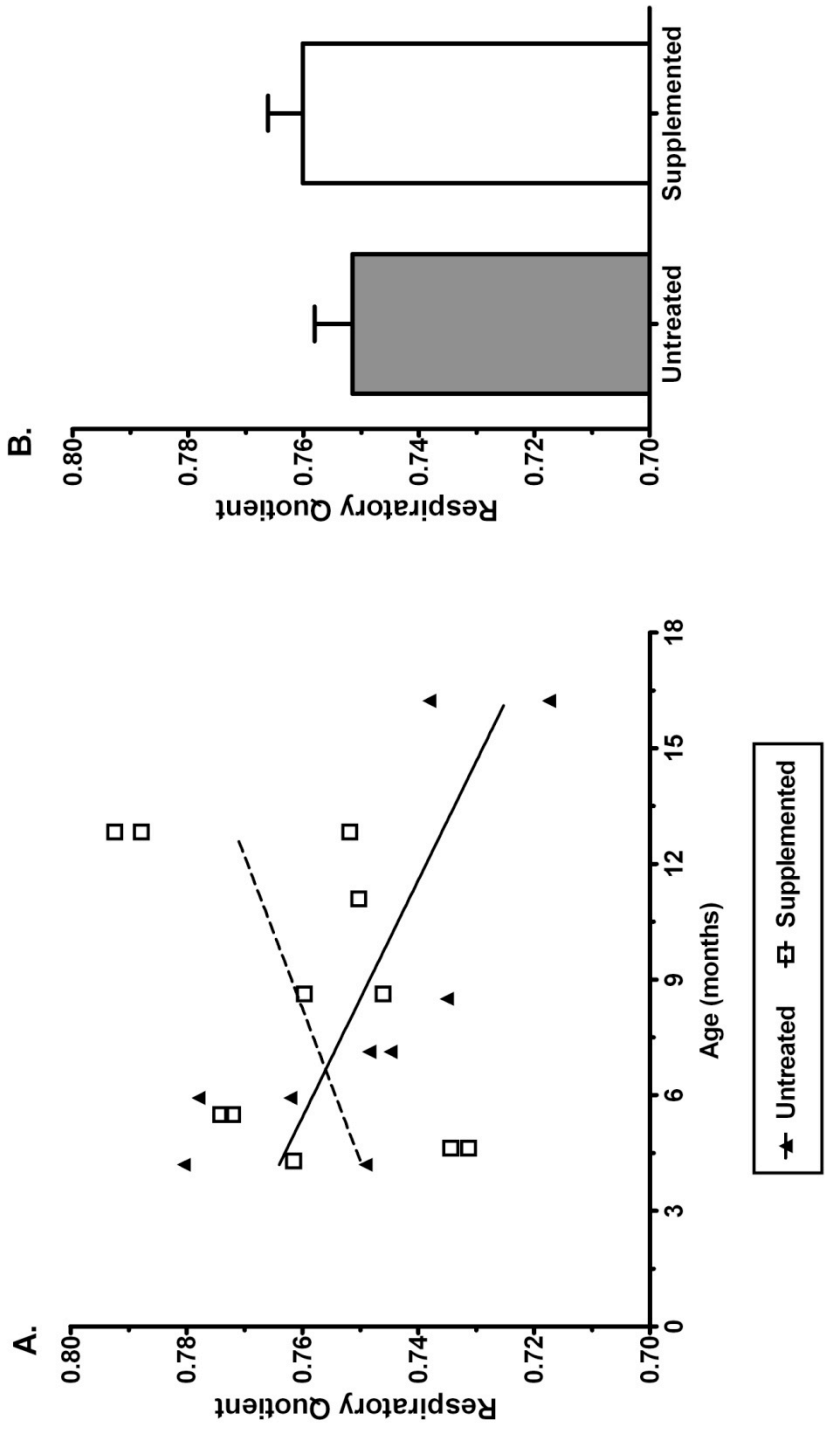

A.
Respiratory Quotient
0.80
0.78
0.76
0.74
0.72
0.70
0
3
6
9
12
15
18
Age (months)
Untreated
Supplemented
B.
Respiratory Quotient
0.80
0.78
0.76
0.74
0.72
0.70
Untreated
Supplemented

Figure 7. Age-related trends and mean resting VO_2 of untreated and supplemented mice in a non-fasting state.

Resting VO_2 of untreated (n = 9; 2-23 months-old) and supplemented (n = 10; 2-27 months-old) mice in a non-fasted state. (A) Comparison of age-related trends of resting VO_2 and (B) differences in mean VO_2 excluding the effects of age. Untreated mice exhibited significant **(-40%; p = 0.0041)** declines in resting VO_2 with age, but remained unchanged in the supplemented group (p = 0.56). Slopes significantly differed **(p = 0.027)**, however mean resting VO_2 did not (p = 0.29).

Linear regressions:

(A.) Untreated: $y = 53.12 - 0.94*x$; $r^2 = 0.71$; **p = 0.0041**

Supplemented: $y = 39.98 - 0.14*x$; $r^2 = 0.37$; p = 0.56

Difference between slopes: **p = 0.027**

Effect of diet: **p = 0.03**

Diet x age interaction: **p = 0.0071**

Summary of means:

(B.) Untreated: 39.70 ± 2.863; n = 9

Supplemented: 37.83 ± 1.866; n = 10

Difference between means: 1.865 ± 3.35, p = n.s.

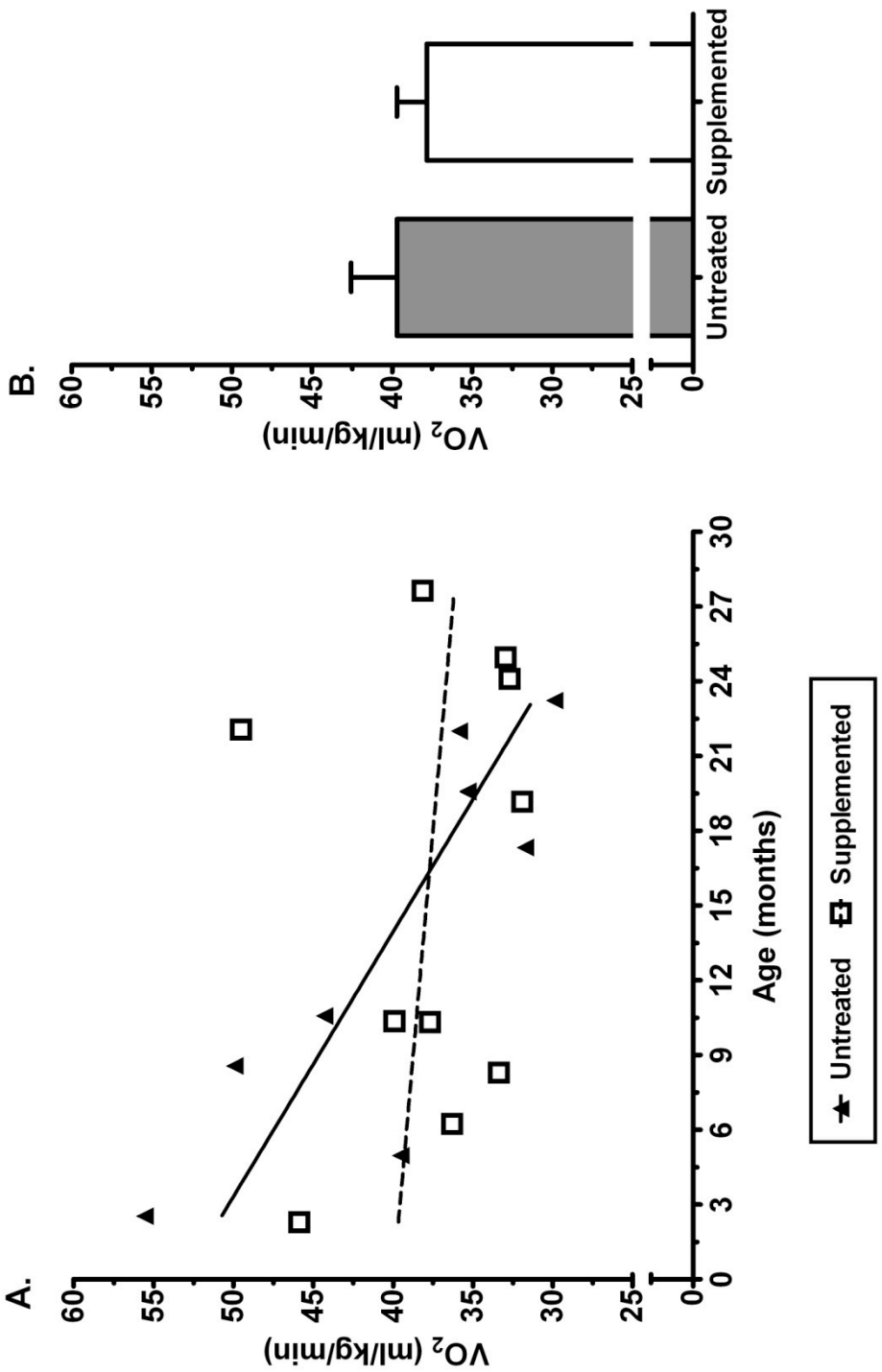

A.
B.
VO2 (ml/kg/min)
Age (months)
Untreated
Supplemented
0
3
6
9
12
15
18
21
24
27
30
25
30
35
40
45
50
55
60

Figure 8. Age-related trends and mean resting respiratory exchange ratio (RER) of untreated and supplemented mice in a non-fasted state.

Resting RER of untreated (n = 9; 2-23 months-old) and supplemented (n = 10; 2-27 months-old) mice in a non-fasted state. (A) Trends in resting RER between diets with respect to age (months) reveal significant age-related declines in untreated mice **(p = 0.013)** while supplemented males remain unchanged. The supplemented regression was significantly elevated over the untreated group, and was reflected in a mean resting RER (B) that was significantly lower in the untreated group **(p = 0.022)**, indicating greater fat utilization.

Linear regressions:

(A.) Untreated: $y = 0.84 - 0.0048*x$; $r^2 = 0.61$; **p = 0.013**

Where x = age (months) and y = respiratory exchange ratio (RER)

Supplemented: $y = 0.85 - 0.0011*x$; $r^2 = 0.52$; p = 0.49

Difference between slopes: p = 0.14

Effect of diet: **p = 0.0091**

Effect of age: **p = 0.041**

Diet x age interaction: **p = 0.042**

Summary of means:

(B.) Untreated: 0.7765 ± 0.01565; n = 9

Supplemented: 0.8285 ± 0.01363; n = 10

Difference between means: -0.05197 ± 0.02066, **p = 0.022**

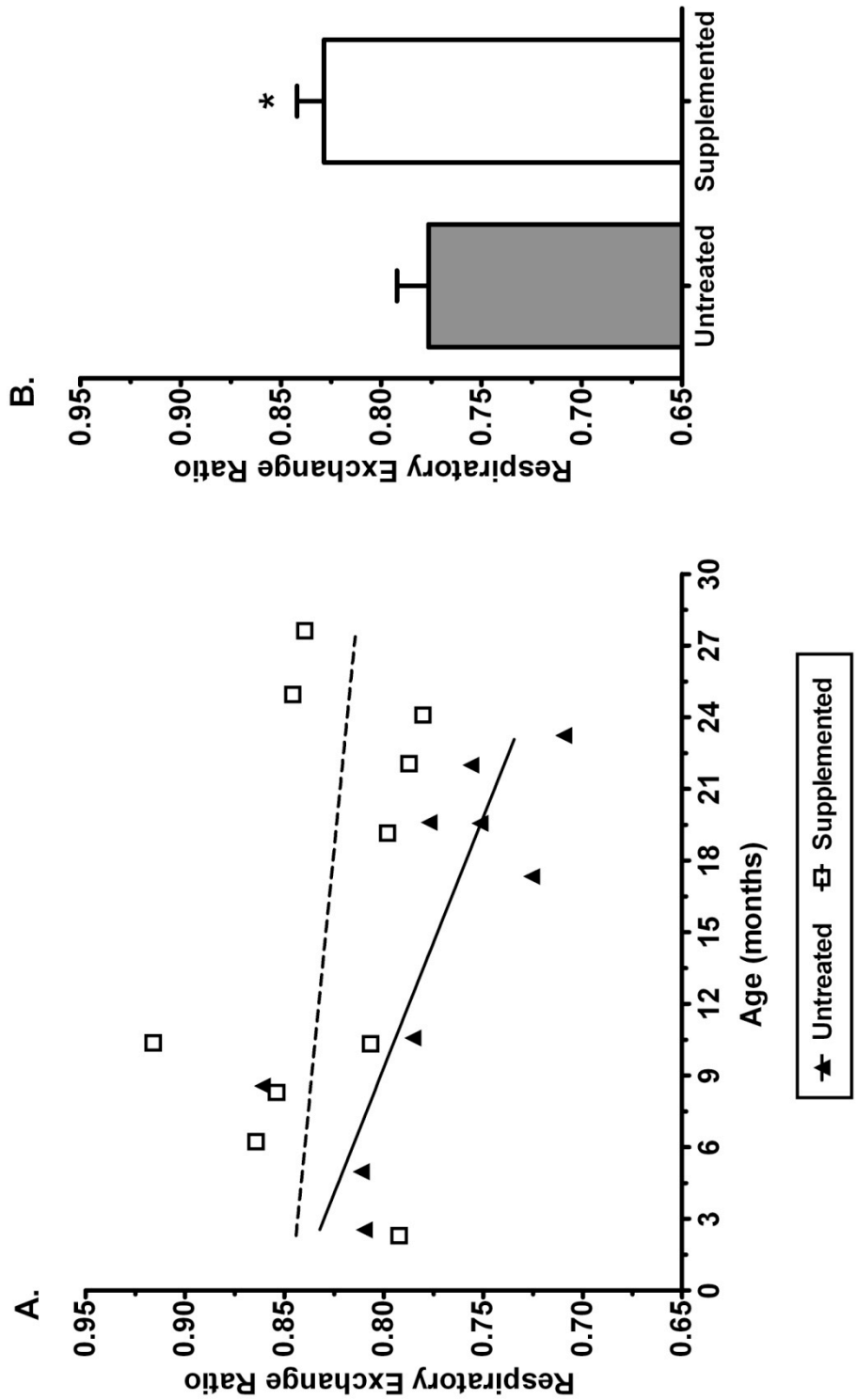

A.
Respiratory Exchange Ratio
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0
3
6
9
12
15
18
21
24
27
30
Age (months)
Untreated
Supplemented
B.
Respiratory Exchange Ratio
0.95
0.90
0.85
0.80
0.75
0.70
0.65
*
Untreated
Supplemented

Figure 9. Effect of diet on 24-hour VO_2 in normal untreated and supplemented male mice.

Effect of diet on 24-hour VO_2 (ml/kg/min) of normal untreated (n = 10; 2-23 months-old) and supplemented (n = 10; 2-28 months-old) male mice with respect to age (months) during 12-hour (A.) photophase and (B.) scotophase. Note that food and water available ad libitum. During both photoperiods only untreated mice show significant age-related declines in VO_2, with the greatest difference between diets seen in young animals.

Linear regressions:

(A) Untreated: $y = 52.94 - 0.9431*x$; $r^2 = 0.43$; **$p = 0.041$**

Supplemented: $y = 35.68 + 0.062*x$; $r^2 = 0.0033$; $p = 0.88$

Difference between slopes: $p = 0.14$

Effect of diet: **$p = 0.077$**

Effect of age: $p = 0.13$

(B) Untreated: $y = 59.37 - 0.84*x$; $r^2 = 0.25$; **$p = 0.014$**

Supplemented: $y = 41.93 + 0.088*x$; $r^2 = 0.0070$; $p = 0.82$

Difference between slopes: $p = 0.16$

Effect of diet: $p = 0.11$

Effect of age: $p = 0.25$

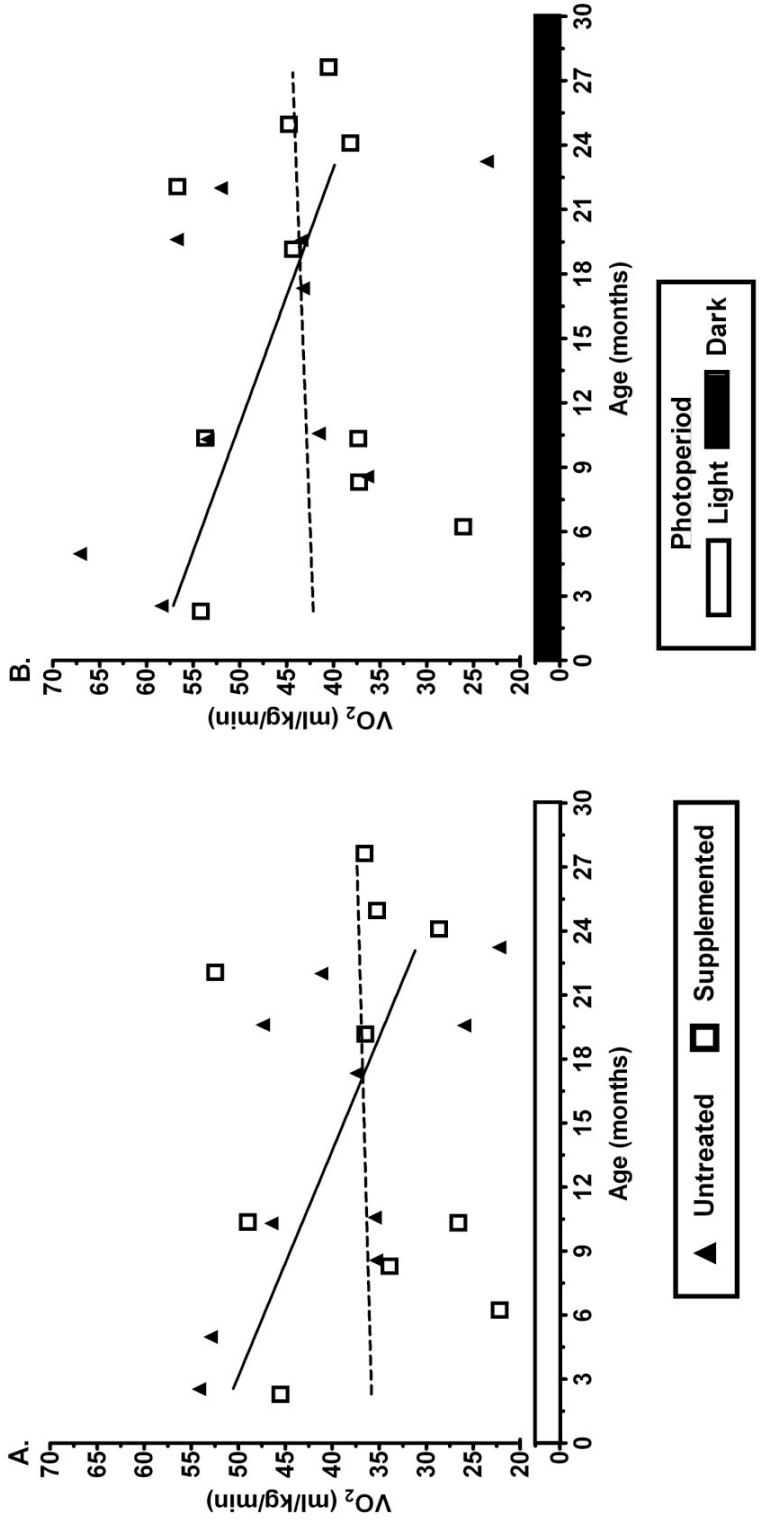

A.
B.
VO2 (ml/kg/min)
Age (months)
Untreated
Supplemented
Photoperiod
Light
Dark

Figure 10. 24-hour VO_2 patterns of untreated and supplemented mice broken down per hour.

Hourly analysis of circadian patterns of VO_2 (ml/kg/min) in (A) pooled age groups of untreated (n = 9; 2-23 months-old) and supplemented (n = 10; 2-27 months-old) mice, (B) only young untreated (n = 4; ≤ 12 months-old) and supplemented (n = 5; ≤ 12 months-old) mice, (C) only old untreated (n = 5; > 12 months-old) and supplemented (n = 5; > 12 months-old) mice and (D) comparison of mean 24-hour VO_2 of pooled, only young and only old age groups. Data are presented as mean ± SEM VO_2 values per hour. Significance: *** ($p < 0.001$) compared to untreated mice. Overhead bars indicate other individual differences.

Summary of Two-way ANOVAs:

(A) Effect of diet: **$p < 0.001$**

Effect of Time: n.s.

Interaction (diet*time): n.s.

(B) Effect of diet: **$p < 0.001$**

Effect of Time: n.s.

Interaction (diet*time): n.s.

(C) Effect of diet: n.s

Effect of Time: n.s.

Interaction (diet*time): n.s.

Summary of mean values:

(D) **Combined age groups:**

Untreated: 45.60 ± 0.7675, n = 9

Supplemented: 39.58 ± 0.9468, n = 10

t-test (effect of diet): **p < 0.001**

Young age group:

Untreated: 51.18 ± 0.8303, n = 4

Supplemented: 41.29 ± 1.167, n = 5

t-test (effect of diet): **p < 0.001**

Old age groups:

Untreated: 41.21 ± 1.190, n = 5

Supplemented: 37.86 ± 1.218, n = 5

t-test (effect of diet): n.s.

Cross comparison (t-test):

Young untreated vs. old untreated: **p < 0.001**

Young supplemented vs. old supplemented: n.s.

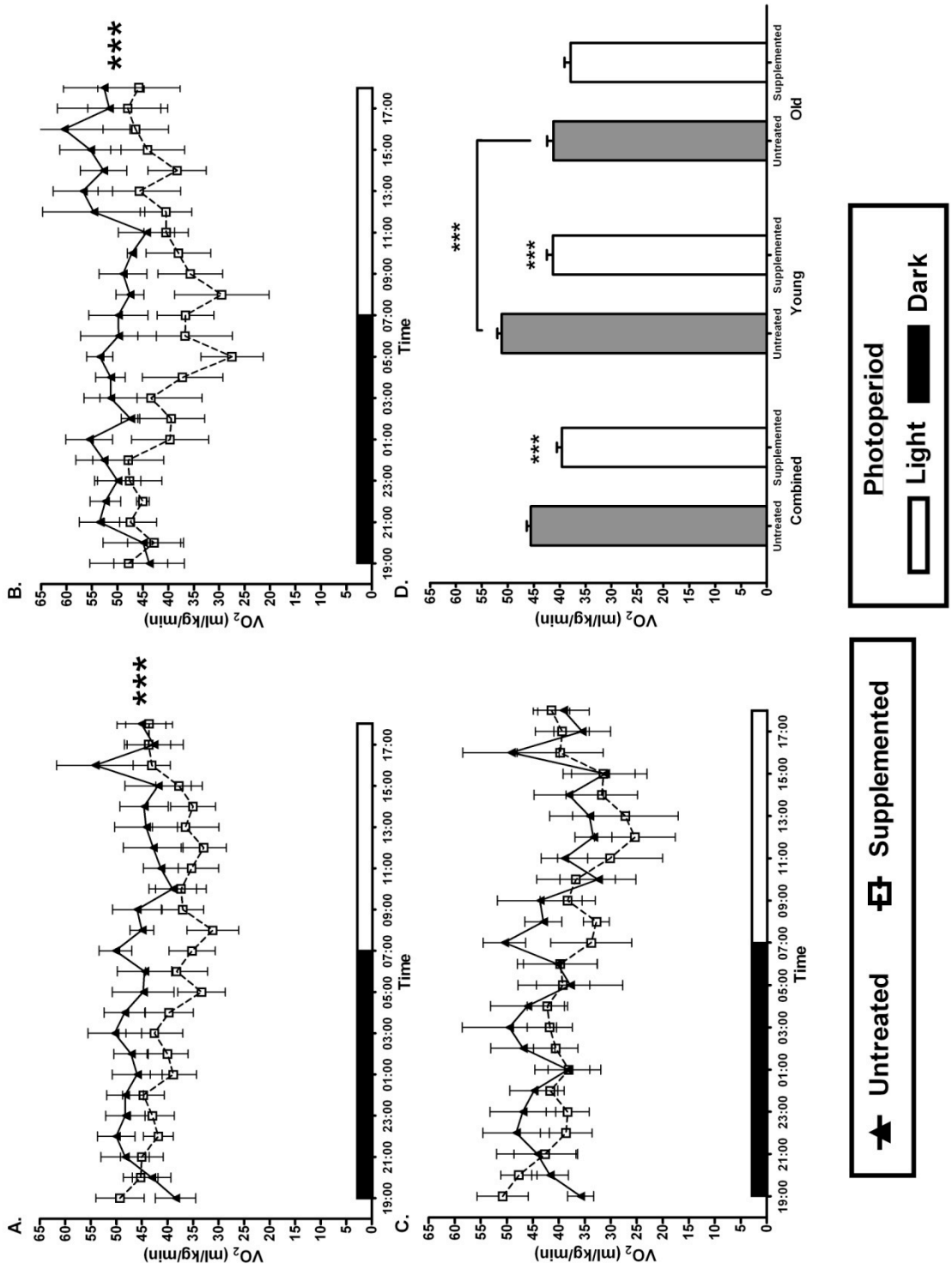

A.
B.
C.
D.
VO_2 (ml/kg/min)
Time
***
Untreated
Supplemented
Combined
Young
Old
Photoperiod
Light
Dark

Figure 11. Effect of diet on relationship between mean hourly VO_2 and spontaneous activity in normal untreated and supplemented male mice.

There is a positive correlation between mean hourly VO_2 (ml/kg/min) and SpA in untreated (n = 9; 2-23 months-old) and supplemented (n = 10; 2-27 months-old) mice that is strongly influenced by diet (**$p < 0.0000001$**). Data represent mean ± SEM of VO_2 and SpA values per hour. Note the significantly elevated regression of untreated mice.

Linear regressions:

Untreated: $y = 30.20 + 0.029*x$; $r^2 = 0.37$; **$p = 0.0018$**

Where x = spontaneous actions (/hr) and y = VO_2 (ml/kg/min)

Supplemented: $y = 24.22 + 0.028*x$; $r^2 = 0.35$; **$p = 0.0022$**

Difference between slopes: $p = 0.92$

Effect of diet: **$p < 0.0000001$**

Effect of SpA: **$p = 0.000009$**

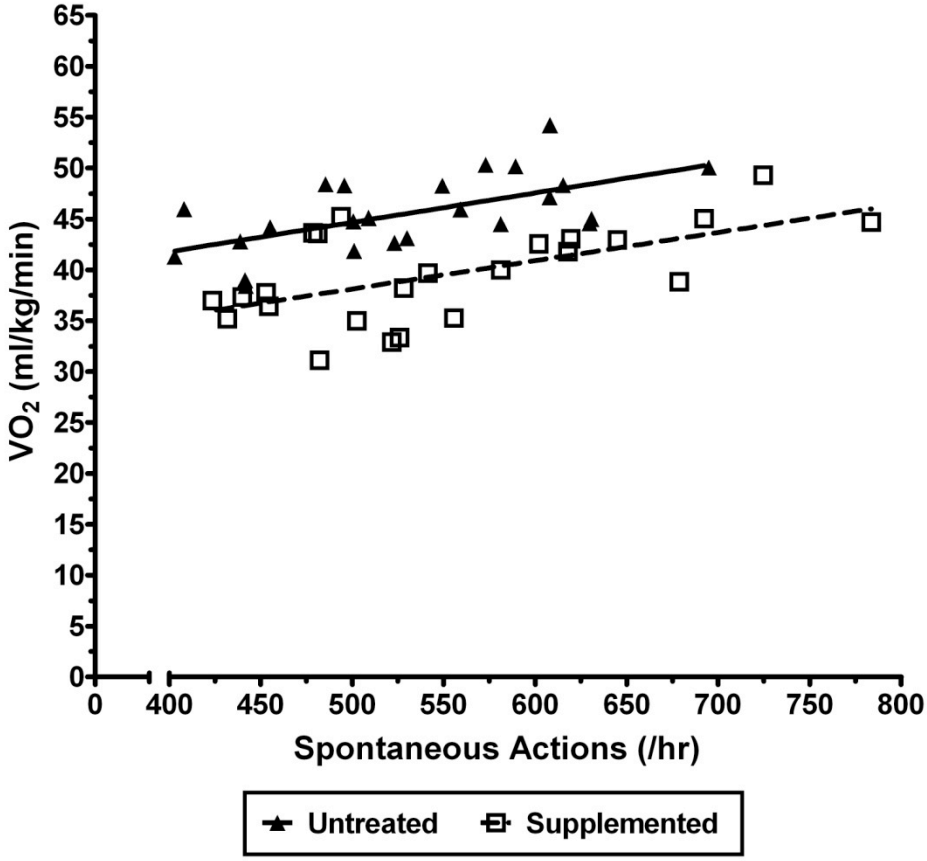

65
60
55
50
45
40
35
30
25
20
15
10
5
0
VO_2 (ml/kg/min)
0
400
450
500
550
600
650
700
750
800
Spontaneous Actions (/hr)
Untreated
Supplemented

Table 2. Gross left ventricular wall thickness measurements of heart tissue samples from normal and transgenic male mice.

A summary of gross morphological analysis of left ventricular (LV) wall thickness in normal (untreated: n = 17, 2-24 months-old; supplemented: n = 13, 2–18 months-old) and transgenic (untreated: n = 12, 2-9 months-old; supplemented: n = 8, 2–20 months-old). Note that for normal mice, LV wall thickness is compared against tibia length (TL), and for transgenic mice body weight (BW) was used instead of TL due to tibia unavailability.

Feature	Mean value		Student *t*-test
	Nr untreated	**Nr supplemented**	
LV wall thickness/TL	0.12 ± 0.012	0.08 ± 0.008	**p = 0.017**
	Tg untreated	**Tg supplemented**	
LV wall thickness/BW	0.065 ± 0.008	0.047 ± 0.004	**P = 0.028**

Figure 12. Effects of diet on age-related levels of H_2O_2 in hearts of normal and transgenic male mice.

Effects of diet on age-related trends of background H_2O_2 (mM/mg) in heart samples from (A) normal (untreated: n = 14, 3-25 months-old; supplemented: n = 12, 7-21 months-old) and (B) transgenic (untreated: n = 12, 4-23 months-old; supplemented: n = 7; 8-15 months-old) male mice.

Linear regressions:

(A.) Untreated: $y = 272.6 – 0.14*x$; $r^2 = 0.0049$; $p = 0.81$

Where x = age (months) and y = H_2O_2 (mM/mg)

Supplemented: $y = 277.4 – 0.21*x$; $r^2 = 0.32$; $p = 0.055$

Difference between slopes: p = 0.33

Effect of diet: n.s.

Effect of age: n.s.

Diet*Age interaction: n.s.

(B.) Untreated: $y = 270.5 + 0.29*x$; $r^2 = 0.19$; $p = 0.15$

Supplemented: $y = 269.7 + 0.38*x$; $r^2 = 0.088$; $p = 0.52$

Difference between slopes: p = 0.87

Effect of diet: n.s.

Effect of age: n.s.

Diet*Age interaction: n.s.

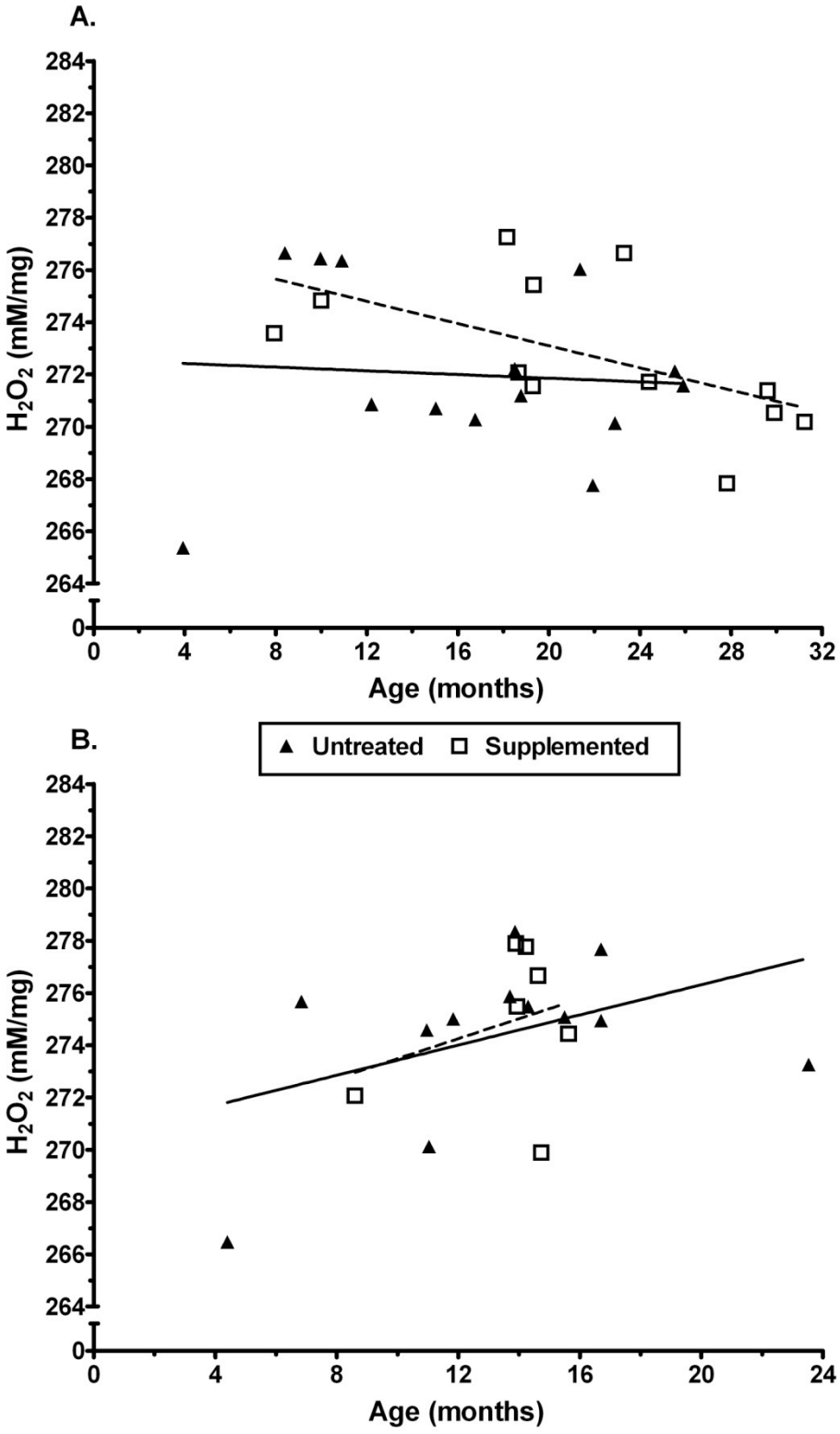

A.
H_2O_2 (mM/mg)
284
282
280
278
276
274
272
270
268
266
264
0
0
4
8
12
16
20
24
28
32
Age (months)
B.
Untreated
Supplemented
H_2O_2 (mM/mg)
284
282
280
278
276
274
272
270
268
266
264
0
0
4
8
12
16
20
24
Age (months)

Figure 13. Mean H_2O_2 levels in hearts of untreated and supplemented normal and transgenic male mice.

Differences in H_2O_2 (mM/mg) background in heart tissue of normal (untreated: n = 14, 3-25 months-old; supplemented: n = 12, 7-31 months-old) and transgenic (untreated: n = 12, 4-23 months-old; supplemented: n = 7, 8-15 months-old) male mice. (A.) Comparison of mean values showed no significant effect of diet in either genotype (Nr: p = 0.54; Tg: p = 0.74). (B.) Overall, transgenic males have significantly higher H_2O_2 levels than normal males (**p = 0.021**).

Summary of means

(A.) Nr **Males**

Untreated: 272.0 ± 0.9044; n = 14

Supplemented: 272.8 ± 0.8177; n = 12

Difference between means: 0.7738 ± 1.237, p = 0.54

Tg Males

Untreated: 274.4 ± 0.9327; n = 12

Supplemented: 274.9 ±1.134; n = 7

Difference between means: 0.5124 ± 1.49, p = 0.74

(B.) Genotype comparison

Untreated: 272.3 ± 0.6089; n = 26

Supplemented: 274.6 ± 0.7050; n = 19

Difference between means: 2.230 ± 0.9331

Genotype effect: **p = 0.021**

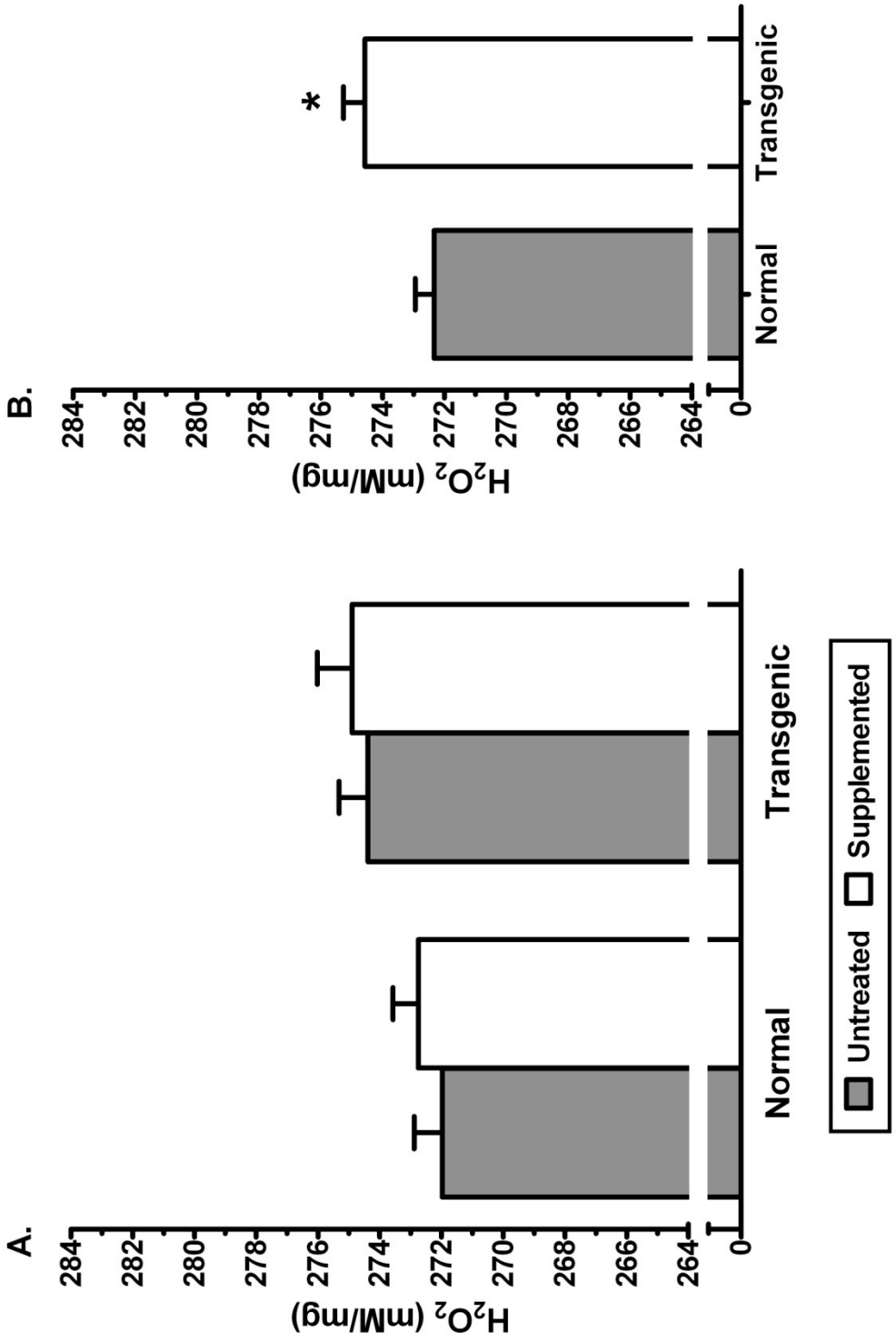

A.
B.
H_2O_2 (mM/mg)
284
282
280
278
276
274
272
270
268
266
264
0
Normal
Transgenic
Untreated
Supplemented
*

Figure 14. Age-related trends of catalase activity in hearts of untreated and supplemented normal and transgenic male mice.

Comparison of age-related trends of catalase (U/ml) activity in heart tissue of (A.) normal untreated (n = 14; 3-27 months-old) and supplemented (n = 8; 2-18 months-old) male mice and (B) transgenic untreated (n = 7; 5-15 months-old) and supplemented (n = 10; 2-16 months-old) male mice. Data are presented as mean ± SEM units of catalase activity per animal.

Linear regressions:

(A.) Untreated: $y = 3.54 – 0.013*x$; $r^2 = 0.02$; p = n.s.

Where x = age (months) and y = catalase (U/ml)

Supplemented: $y = 5.69 – 0.13*x$; $r^2 = 0.56$; **p = 0.03**

Difference between slopes: p = **0.039**

Effect of diet: **p = 0.008**

Effect of age: **p = 0.015**

Diet*Age interaction: **p = 0.03**

(B.) Untreated: $y = 2.45 – 0.038*x$; $r^2 = 0.04$; p = n.s.

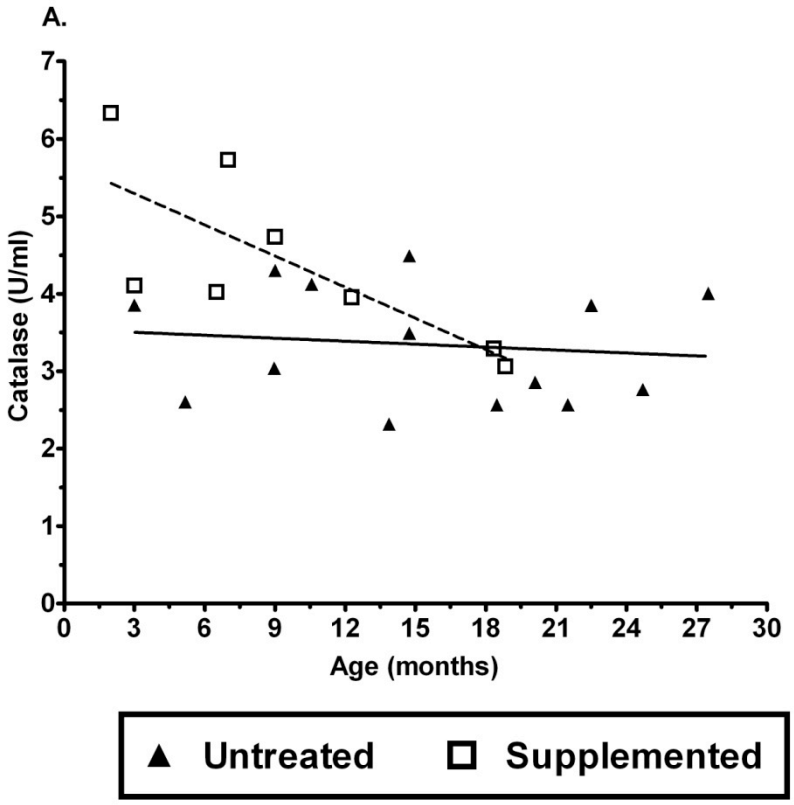
Supplemented: $y = 4.13 – 0.15*x$; $r^2 = 0.44$; **p = 0.035**

Difference between slopes: p = **0.039**

Effect of diet: n.s

Effect of age: **p = 0.035**

Diet*Age interaction: n.s.


A.
Catalase (U/ml)
0
1
2
3
4
5
6
7
0
3
6
9
12
15
18
21
24
27
30
Age (months)
Untreated
Supplemented

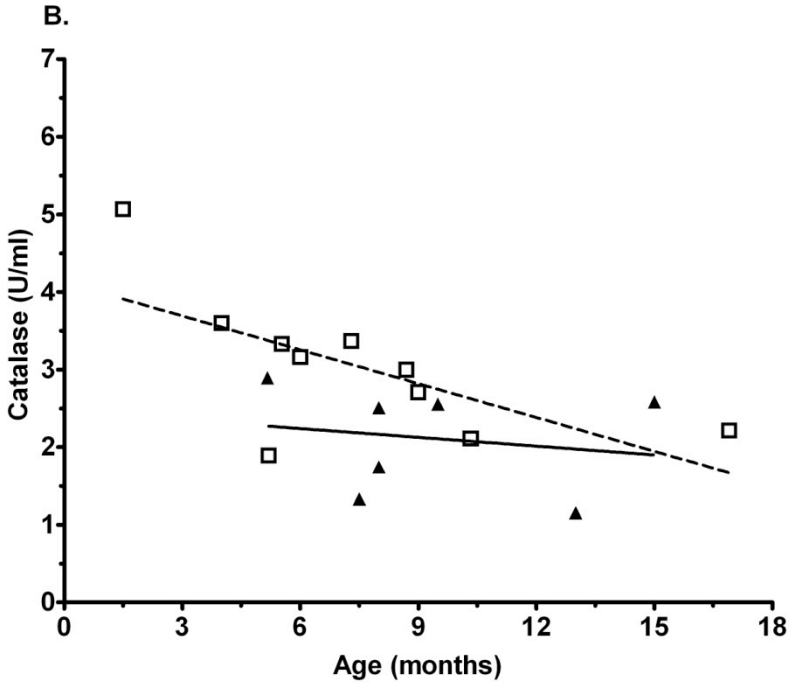

B.
Catalase (U/ml)
0
1
2
3
4
5
6
7
0
3
6
9
12
15
18
Age (months)

Figure 15. Comparison of mean catalase activity in hearts of untreated and supplemented normal and transgenic male mice.

Comparison of mean catalase (U/ml) activity in heart tissue of (A.) normal (untreated: n = 14, 3-27 months-old; supplemented: n = 8, 2-18 months-old) and transgenic (untreated: n = 7, 5-15 months-old; supplemented: n = 10, 2-16 months-old) male mice and (B) normal and transgenic genotypes (pooled diet groups). Data are presented as mean ± SEM units of catalase activity per diet group or genotype when appropriate. Significance: * ($p < 0.05$), ** ($p < 0.01$), individual differences are indicated by overhead bars.

Summary of means:

(A.) **Normal mice**

Untreated: 3.345 ± 0.2001; n = 14

Supplemented: 4.405 ± 0.4023; n = 8

Difference between means: 1.060 ± 0.4007

Effect of diet: **p = 0.016**

Transgenic Mice

Untreated: 2.109 ± 0.2609; n = 7

Supplemented: 3.044 ± 0.2907; n = 10

Difference between means: 0.9348 ± 0.4113

Effect of diet: **p = 0.038**

Cross-comparison (student t-test)

Untreated: Normal vs. Transgenic, **p = 0.0017**

Supplemented: Normal vs. Transgenic, **p = 0.013**

(B.) **Genotype comparison**

Normal: 3.730 ± 0.2185; n = 22

Transgenic: 2.659 ± 0.2273; n = 17

Difference between means: 1.071 ± 0.3190

Effect of genotype: **p = 0.0018**

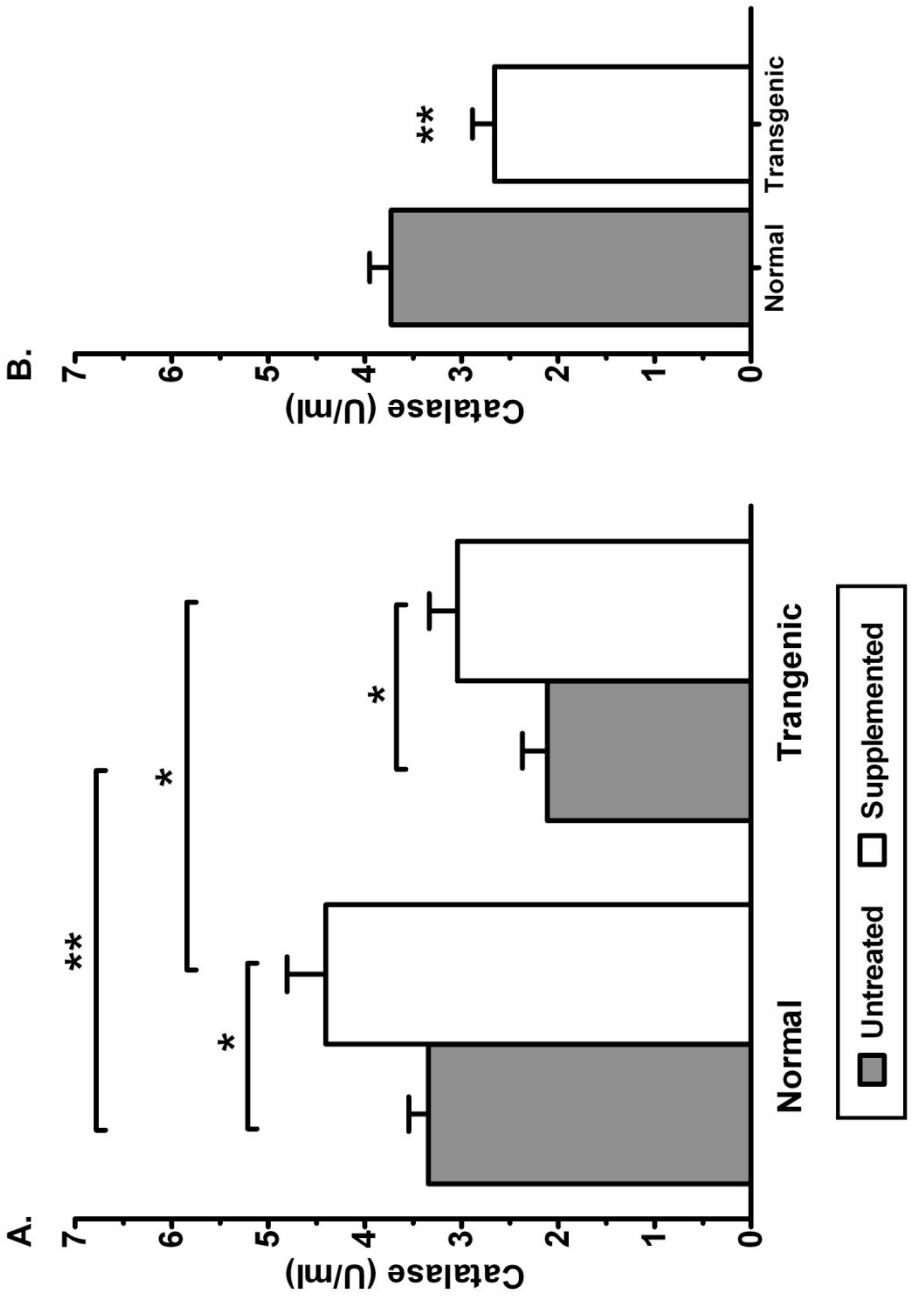

A.
Catalase (U/ml)
0
1
2
3
4
5
6
7
**
*
*
*
Normal
Trangenic
Untreated
Supplemented
B.
Catalase (U/ml)
0
1
2
3
4
5
6
7
**
Normal
Transgenic

Figure 16. Effects of diet on age-related trends of background H_2O_2 in kidneys of normal and transgenic male mice.

Effects of diet on age-related trends of background H_2O_2 (mM/mg) in kidney samples from (A) normal (untreated: n = 19, 3-29 months-old; supplemented: n = 14, 4-23 months-old) and (B) transgenic (untreated: n = 12, 4-16 months-old; supplemented: n = 10; 5-15 months-old) male mice. Note the opposite age-related trends observed in untreated mice (**p = 0.0040**).

Linear regressions:

(A.) Untreated: $y = 282.2 – 0.19*x$; $r^2 = 0.26$; **p = 0.025**

Where x = age (months) and y = H_2O_2 (mM/mg)

Supplemented: $y = 273.2 + 0.35*x$; $r^2 = 0.29$; **p = 0.045**

Difference between slopes: p = **0.0040**

Effect of diet: n.s.

Effect of age: **p = 0.0032**

Diet*Age interaction: n.s.

(B.) Untreated: $y = 281.3 – 0.34*x$; $r^2 = 0.28$; p = 0.076

Supplemented: $y = 278.3 - 0.12*x$; $r^2 = 0.043$; p = 0.56

Difference between slopes: p = 0.42

Effect of diet: n.s.

Effect of age: n.s.

Diet*Age interaction: n.s.

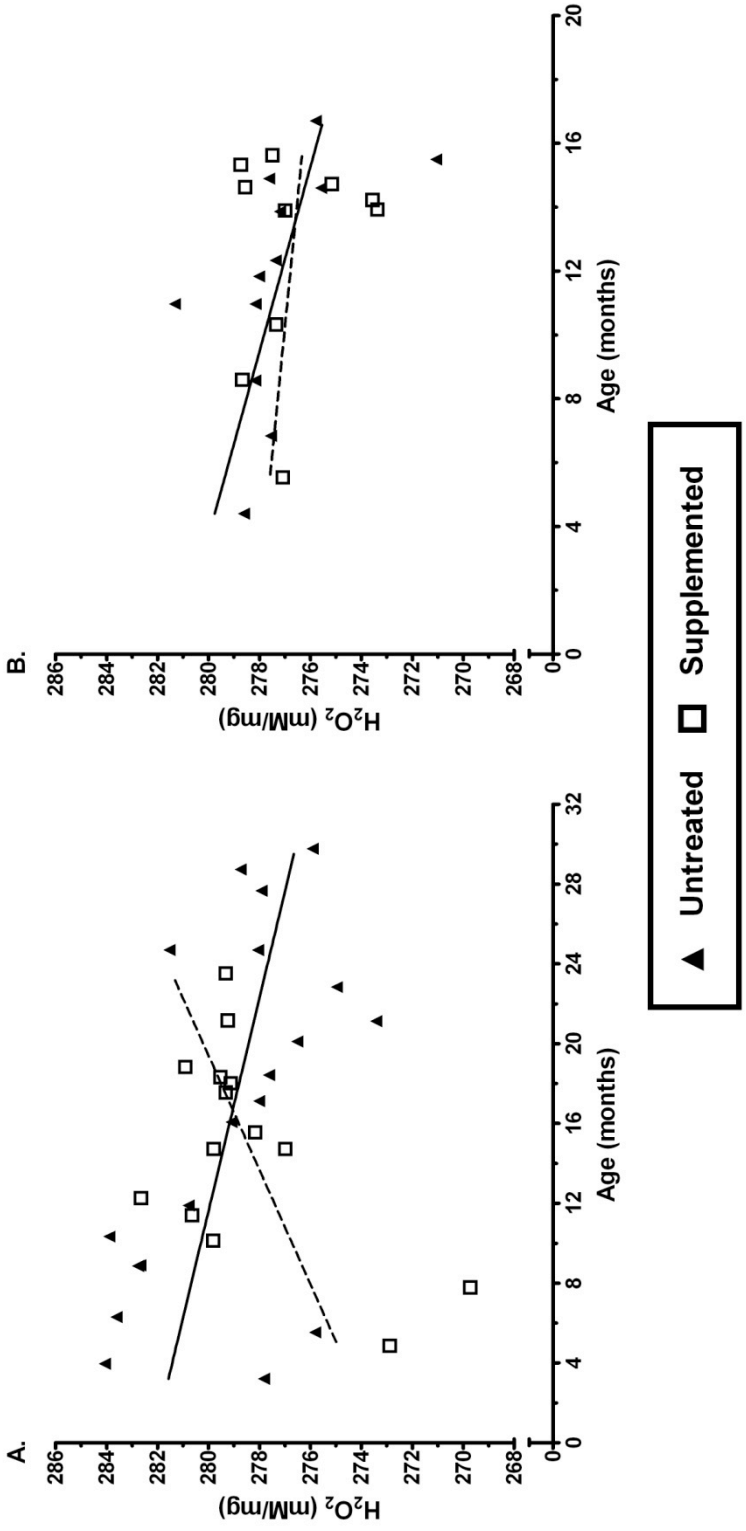

A.
B.
H_2O_2 (mM/mg)
Age (months)
Untreated
Supplemented

Figure 17. Effect of diet on mean H_2O_2 in kidneys of normal and transgenic male mice.

Effects of diet on mean H_2O_2 (mM/mg) background in kidney samples from (A.) normal male (untreated: n = 19, 3-29 months-old; supplemented: n = 14, 4-23 months-old) and transgenic (untreated: n = 12, 4-16 months-old; supplemented: n = 10; 5-15 months-old) male mice, and (B.) comparison of genotypic differences with pooled diet groups. Data are reported as mean ± SEM background H_2O_2 (mM/mg) for diet and genotypic group. Significance: * ($p < 0.05$) compared with normal males.

Summary of means:

(A.) **Normal males**

Untreated: 279.1 ± 0.9327; n = 19

Supplemented: 278.4 ± 0.8929; n = 14

Difference between means: 0.6813 ± 1.154

Effect of diet: p = 0.87

Transgenic males

Untreated: 277.2 ± 0.6961; n = 12

Supplemented: 276.7 ± 0.6336; n = 10

Difference between means: 0.5048 ± 0.9579

Effect of diet: p = 0.60

(B.) **Genotype comparison**

Normal: 278.8 ± 0.5643; n = 33

Transgenic: 277.0 ± 0.4687; n = 22

Difference between means: 1.853 ± 0.7910

Effect of genotype: **p = 0.023**

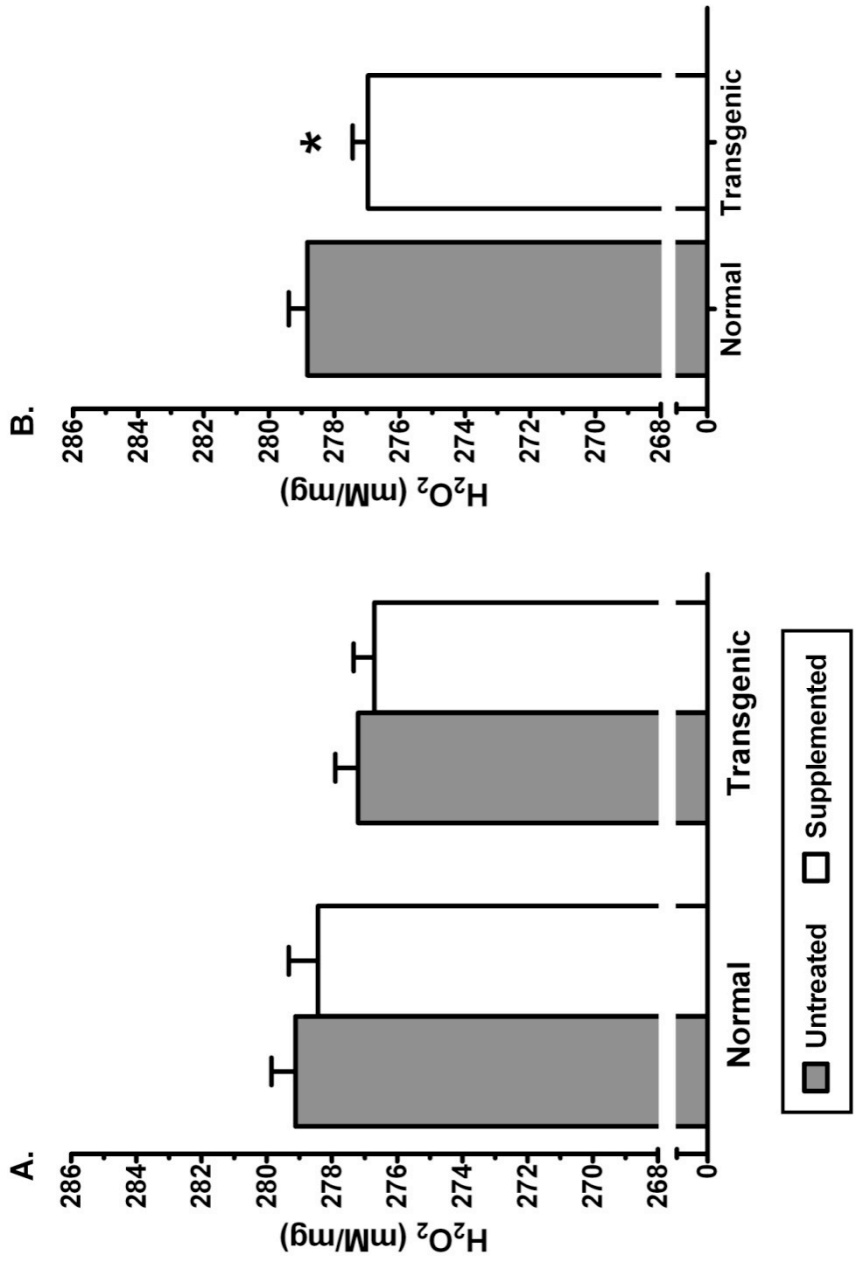

A.
B.
H_2O_2 (mM/mg)
286
284
282
280
278
276
274
272
270
268
0
Normal
Transgenic
Untreated
Supplemented
*

Figure 18. Comparison of mean catalase activity in kidneys from untreated and supplemented normal and transgenic male mice.

Comparison of mean catalase (U/ml) activity in kidney samples from (A.) normal (untreated: n = 14, 3-29 months-old; supplemented: n = 14, 3-23 months-old) and transgenic (untreated: n = 13, 4-16 months-old; supplemented: n = 13, 5-15 months-old) male mice and (B) normal and transgenic genotypes (pooled diet groups). Data are presented as mean ± SEM units of catalase activity per diet group or genotype where appropriate. Significance: * ($p < 0.05$), *** ($p < 0.001$)

Summary of means:

(A.) **Normal mice**

Untreated: 2.654 ± 0.2429; n = 13

Supplemented: 2.291 ± 0.2302; n = 13

Difference between means: 0.3637 ± 0.3346

Effect of diet: p = 0.29

Transgenic Mice

Untreated: 3.388 ± 0.1688; n = 14

Supplemented: 3.479 ± 0.1375; n = 14

Difference between means: 0.09134 ± 0.2177

Effect of diet: p = 0.68

Cross-comparison (student t-test)

Untreated: Normal vs. Transgenic, **p = 0.022**

Supplemented: Normal vs. Transgenic, **p = 0.0002**

(B.) **Genotype comparison**

Normal: 2.473 ± 0.1679; n = 26

Transgenic: 3.434 ± 0.1070; n = 28

Difference between means: 0.9612 ± 0.1070

Effect of genotype: **$p < 0.0001$**

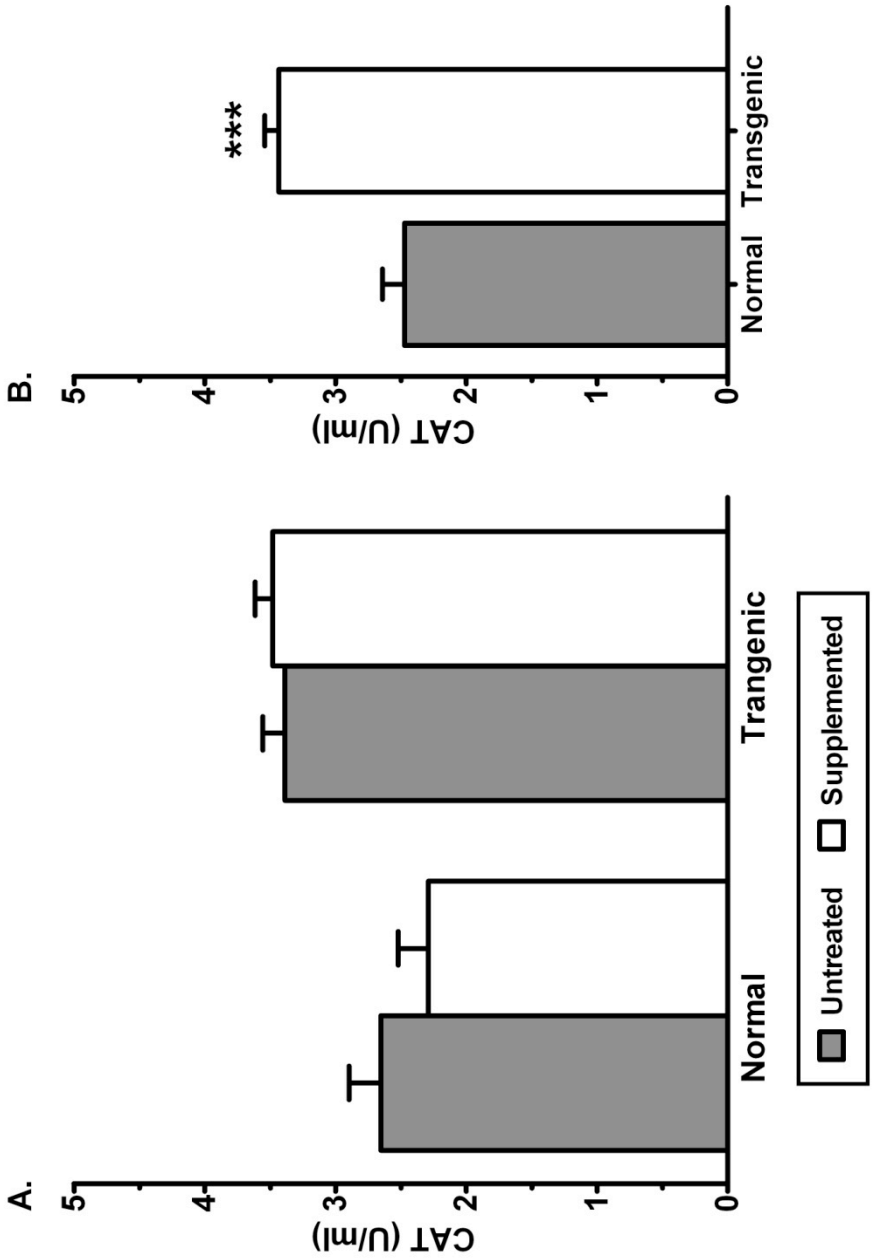

A.
CAT (U/ml)
0
1
2
3
4
5
Normal
Trangenic
Untreated
Supplemented
B.
CAT (U/ml)
0
1
2
3
4
5
***
Normal
Transgenic

Figure 19. Comparison of the glutathione ratio (reduced:oxidized) in urine samples from old untreated and supplemented normal male mice.

Comparison of mean GSH:GSSG ratio in urine samples from old normal male mice (untreated: n = 12, ≥ 12 months-old; supplemented: n = 9; ≥ 12 months-old). Data are presented as mean ± SEM GSH:GSSG ratio by diet group. Significance: *** ($p < 0.001$) compared with untreated.

Summary of means:

Normal male mice

Untreated: 0.6471 ± 0.05502; n = 12

Supplemented: 0.9266 ± 0.01567; n = 9

Difference between means: 0.2795 ± 0.06534

Effect of diet: **p = 0.0004**

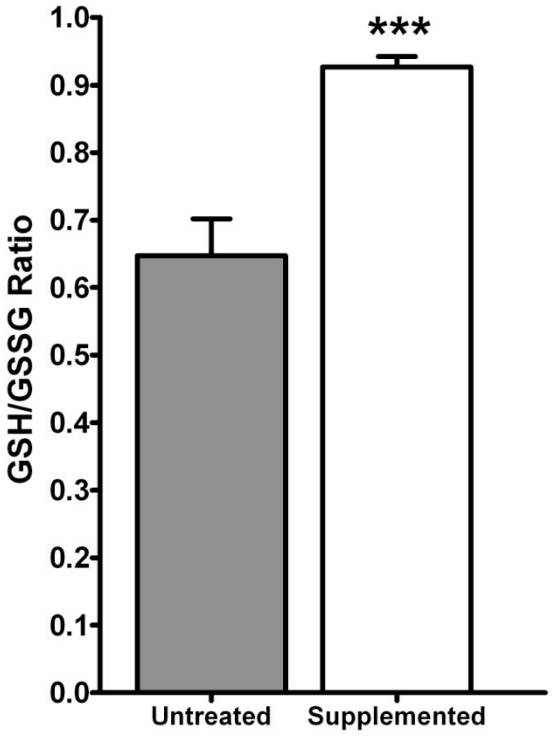

GSH/GSSG Ratio
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
Untreated
Supplemented

Figure 20. Intra-peritoneal glucose tolerance test (IPGTT) in old untreated and supplemented male mice

Effect of diet on whole-body glucose tolerance in old (≥ 12 months-old) normal male (untreated, n = 12; supplemented, n = 9) mice. Blood glucose mmol/L values are presented as mean ± SEM per diet group. Significance: ** ($p < 0.01$) versus untreated.

Area under curve (AUC) summary

Old untreated males:

AUC: 779.4 ± 32.83 mmol/L per 120 min

% Area: 100%

Peak X = 30 min

Peak Y = 15.98 mmol/L

Old Supplemented males:

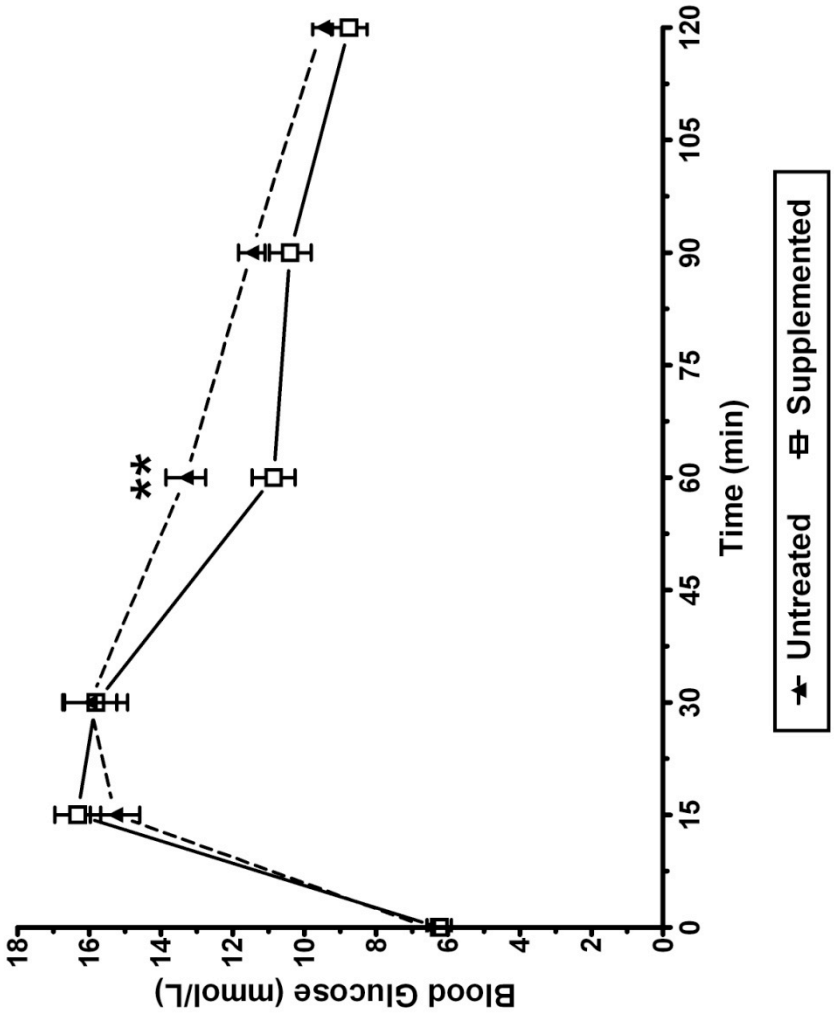
AUC: 676.2 ± 39.14 mmol/L per 120 min

% Area: 100%

Peak X = 15 min

Peak Y = 16.32 mmol/L

Difference between AUC: **p = 0.056**


Blood Glucose (mmol/L)
0
2
4
6
8
10
12
14
16
18
0
15
30
45
60
75
90
105
120
Time (min)
**
Untreated
Supplemented

CHAPTER FOUR: DISCUSSION

4.1 Spontaneous physical activity (SpA)

Declining physical activity with age is one of the most reliable biomarkers of aging in human and animal models including *Drosophila melanogaster* and rodents (Ingram, 2000; Le Bourg, 1987; Longo et al., 2010; Sallis, 1999). Regardless, this remains poorly understood. For humans the focus is on subjects >60 years-old, however, cross-sectional data suggests that the most significant declines in physical activity are actually seen in youthful ages (13–18 years) with males exhibiting the greatest declines (Caspersen, C.J., Merritt, R.K, Stephens T., 1994; Telama & Yang, 2000; van de Laar et al., 2011). A similar trend is apparent in rodents, where physical activity declines linearly and up to 50% through adult ages (Ingram, 2000). This highlights the importance of developing effective interventions to both attenuate progressive adolescent declines in locomotor activity and to elevate levels of physical activity in the elderly (see also Aksenov et al. 2010). This is particularly relevant for aging since physical activity is positively associated with health and life-span (N. Owen, Healy, Matthews, & Dunstan, 2010).

Our laboratory previously found substantial declines (>50%) in daily locomotion (i.e., excluding exercise) even in youth that progressed steadily beyond 24 months of age in untreated normal mice (Aksenov et al., 2010). By comparison, supplemented mice showed virtually no loss in locomotor activity (Aksenov et al., 2010). Therefore, we hypothesized that supplemented Nr mice would maintain youthful levels of total daily activity with age (SpA), and expected to see a significant decline in untreated controls.

Here we used a simple, yet highly accurate gravimetric approach to evaluate total activity during 24-hour calorimetry studies (see Biesiadecki, et al. 1999) (Biesiadecki et al., 1999). The DSP maintained activity levels with age in supplemented Nr consistent with our predictions, while untreated mice showed a significant loss (-17%, **fig. 1a**) of SpA between 3 and 24 months. Several ingredients in the DSP are known to increase physical activity in old rodents. Wolden-Hanson et al. (2000) showed that 12 weeks of daily melatonin supplementation (0.4 μg/ml) in middle-aged rats offset age-associated decreases in pineal melatonin secretion, reduced body fat by 16% and enhanced locomotor activity by 19% while maintaining youthful levels of food intake, plasma leptin and insulin (Wolden-Hanson et al., 2000). Furthermore, a combination of acetyl-L-carnitine and α-lipoic acid boosted physical activity in young rats, partially restored ambulatory behavior and generally protected against mitochondrial dysfunction in old rats (Hagen et al., 1998; Hagen et al., 2002; Liu, Head, Kuratsune, Cotman, & Ames, 2004). Further benefits may arise via synergistic interactions of other ingredients in our DSP.

Our methodology did not measure variation in intensity of activity, whereas resting was recognized as periods of absolutely no movement (sampling rate: once every 5 seconds). Thus resting is a more comparable measure of periods of reduced locomotor costs. Resting also provides a relative estimate of sleep. Sleep may be a critical aspect for aging as different periods of sleep may be differentially associated with both GH axis signaling to the target of rapamycin (that is associated with accelerated aging) and subsequent activity of the forkhead transcription factors (associated with stress resistance and reduced aging rates) (Rollo, 2010). By 24 months, untreated Nr mice spent 40% (**fig.**

3a) more time resting (hours per day) compared to age-matched supplemented mice. Age-related increases in sedentary behavior and sleeping are strong indicators of impaired energy balance (Ingram, 2000). Our previous work showed substantial declines in striatal neuropeptide Y (NPY) mRNA in old (~1.5-2 years) untreated Nr but not age-matched supplemented Nr mice (Aksenov et al., 2010). NPY in the arcuate nucleus of the hypothalamus potently regulates feeding and energy homeostasis (Manini, 2010). Our findings are consistent with other work showing that arousal (i.e. foraging for food) can increase daily energy expenditure by 10% in rodents (Ravussin et al., 1986). Furthermore, the motivation to expend energy is regulated through dopaminergic systems in the brain (Roth & Joseph, 1994). Chaudhry et al. (2008) showed that striatal dopamine was reduced in lethargic Tg compared to Nr mice, and was elevated in hyperactive circling mice (Chaudhry, Marsh-Rollo, Aksenov, Rollo, & Szechtman, 2008). Studies involving the direct injection of dopamine into the brains of rats showed that the exploratory activity of young rats increased, but this was not observed in older rats (Cousin, Uretsky, & Gerald, 1985). The blunted response to dopamine was attributable to age-related declines in D2 dopamine receptors in old rats (Roth & Joseph, 1994). Overall, these results suggest our DSP may preserve neuromodulatory regulation associated with physical activity, which is vital for quality of life and independence. We conclude that the DSP shows great potential in promoting healthy aging through the maintenance of functional performance with age.

4.2 Indirect calorimetry

Understanding changes in basal metabolic rate (BMR) with age can provide valuable insight regarding energy metabolism and the use of metabolic fuels. The DSP was shown to prevent age-related declines in neuromodulatory factors that regulate feeding and physical activity, both of which influence metabolic rate. Therefore, we hypothesized that supplemented mice will maintain basal metabolic rate, as reflecting in youthful patterns of fasting oxygen consumption and respiratory quotient. Measurements were taken in untreated and supplemented male mice (~3-15 months old) fasted overnight. We found that our DSP significantly altered fasting oxygen consumption in supplemented animals with respect to age, as significantly opposite age-related trends were resolved ($p = 0.018$, **fig. 5a**). At ~3 months of age supplemented mice consumed approximately 25% less oxygen compared to age-matched untreated Nr, but showed significant increases with age (+19%, $b = 0.52$; $p = 0.048$). By comparison untreated mice showed a trend for declining oxygen consumption with age in the fasted state ($b = -0.59$, $p = 0.13$). Overall, mean oxygen consumption was roughly 10% lower in the supplemented group ($p = 0.045$, **fig. 5b**); but this was largely attributed to differences in young mice. In addition, mean respiratory quotient (RQ) between diets did not differ (**fig. 6b**), but we observed significant differences in age-related trends of RQ ($p = 0.010$, ANOCVA; **fig. 6a**). The supplemented mice showed a trend for increasing RQ (+3%, $p = 0.16$), and the untreated group exhibited a significant age-related decline (-5%, $p = 0.021$). These trends resemble our previous results for mitochondrial complex III (see **fig.**

21) and IV activity in brain, with age regressions exhibiting an "X" shaped pattern (Aksenov et al. 2010, 2011).

Non-fasted resting oxygen consumption and respiratory exchange ratios (RER) of untreated and supplemented mice showed similar patterns as for their respective basal metabolic measurements. Although mean resting VO_2 did not differ with diet (**fig. 7b),** significant opposite age-related trends were resolved ($p = 0.027$, **fig 7a**) highlighting a significant effect of the DSP. Resting VO_2 remained unchanged with age in supplemented mice ($p = 0.56$; **fig. 7a**), while young untreated mice consume ~30% more oxygen compared to age-matched supplemented mice, and showed a significant 40% decline with age. The effect of diet was also significant for resting RER values across age ($p = 0.0091$, **fig 8a**), with a significantly higher (+10%, $p = 0.022$; **fig. 8b**) mean resting RER in supplemented compared to untreated mice.

We suspect that the consistently elevated resting RER in supplemented mice reflects increased carbohydrate oxidation. Carbohydrates yield more ATP per mole of O_2 than proteins or lipids (Weber, 2011), and may lower VO_2 observed in supplemented mice compared to age-matched untreated controls. In addition, the higher GSH:GSSG ratio in urine from supplemented mice (discussed below) in comparison to untreated controls indicates this change was not associated with elevates ROS.

Age-related declines in metabolism of Nr untreated mice may reflect multiple causes. Changes in body composition (i.e. decreased fat-free mass and increased fat mass) are widely documented in elderly individuals and these correlate with reductions in metabolic rate (Bosy-Westphal et al., 2003; Kyle et al., 2001; Piers, Soares, McCormack,

& O'Dea, 1998). In particular, the loss of skeletal muscle mass with age (i.e. sarcopenia) contributes to declines in fat-free mass (Piers et al., 1998). Although we do not have measures of body-composition, sarcopenia (another biomarker of aging) is associated with significant declines in physical activity (Krause, McIntosh, & Vallis, 2011), and is characteristic of untreated mice (**figs. 1-3**), as well as aging Tg (Aksenov et al., 2010). The oxygen consumption patterns observed in untreated mice relate to findings of a study assessing physical performance and energy expenditure in a mouse model of accelerated senescence, which reports a 19% decline in oxygen consumption with age (Haramizu, Ota, Hase, & Murase, 2011). Therefore, the pattern of oxygen consumption across age in DSP supplemented mice may be associated with amelioration of age-associated declines in physical activity, and begs the question of how activity and oxygen consumption correlate in the two groups of mice (see **fig. 11,** discussed below).

We hypothesized that supplemented mice would maintain youthful levels of 24-hour oxygen consumption compared to untreated controls. Given the link between oxygen demand and physical activity, this prediction was derived from our previous result showing supplemented mice maintained spontaneous daily locomotor activity with age (Aksenov et al., 2010). A breakdown of 24-hour oxygen consumption patterns (**fig. 9a-b**) shows very similar patterns to resting VO_2 (**fig. 7a**), with the expected diurnal changes (+15%) in oxygen consumption during the scotophase (associated with most locomotor activity, **fig. 9b**) compared to the photophase (**fig. 9a**). Further breakdown of 24-hour oxygen consumption on an hourly basis (**fig. 10a**) shows a highly significant effect of diet ($p < 0.001$), with supplemented mice consuming less oxygen (-12%, $p < 0.001$; **fig. 10d**)

over 24-hours. Also, mean 24-hour oxygen consumption in young (<12 months-old) untreated mice was approximately 24% ($p < 0.001$**, fig, 10d**) higher than young supplemented mice, while no significant differences were found for diet in older (≥ 12 months-old) mice.

These findings suggest that the DSP has a more profound effect on younger mice with regards to differences in oxygen consumption. The increase in oxygen consumption of untreated mice associated with lower activity levels compared to supplemented animals suggests the DSP lowers oxygen demand for activity. We found a positive correlation between mean hourly VO_2 and hourly spontaneous activity levels with a highly significant effect of diet ($p < 0.0000001$, **fig. 11**). For any comparable amount of physical activity, untreated mice consumed ~20% more oxygen and overall had ~12% lower maximal hourly activity levels (untreated: ~700 actions per hour vs. supplemented: ~800 actions per hour). Non-exercise physical activity can account for ~25% of daily energy expenditure, therefore age-related declines in activity levels (reduced daily energy expenditure) parallel changes in metabolic rate with age (Manini, 2010). The reduced oxygen consumption associated with increased physical activity in supplemented mice suggests the DSP may improve metabolic efficiency. This holds promise for enhancing athletic performance. Components of the supplement are known to enhance metabolic efficiency, such as omega-3 fatty acids which lower whole-body oxygen consumption during cycling performance (55% VO_{2max}) in well trained athletes (Peoples, McLennan, Howe, & Groeller, 2008). In addition, a mixed dietary supplement that included α-lipoic acid, acetyl-L-carnitine and coenzyme Q10 (also in our DSP) improved exercise

performance to exhaustion after four weeks (Sun et al., 2011). This was associated with increased mitochondrial complex I, II and III activity in skeletal muscle and transcription factors associated with mitochondrial biogenesis (Sun et al., 2011).

The higher VO_2 in youth and likely higher ROS may explain the rapid deterioration of mitochondrial function in aging untreated mice compared to supplemented animals (Aksenov et al. 2010, 2011). Mitochondrial content in human skeletal muscle declines by ~25% with age, as does the efficiency of remaining mitochondria (Conley et al., 2007). Malatesta et al. (2003) found that age-related declines in skeletal muscle mitochondrial content and efficiency translate into a 30% increased energetic cost of daily activities such as walking (Malatesta et al., 2003). These results are consistent with our prediction that the DSP maintains aspects of daily energy expenditure and metabolism with age, also showing potential as an ergogenic aid in athletic performance. We conclude that our complex dietary supplement may generally promote better quality of life in older ages by ameliorating age-related declines in physical activity and energy metabolism.

4.3 Whole-tissue H_2O_2 background and catalase activity

4.3.1 Heart tissue

The heart represents an ideal tissue to investigate the potential pathophysiological contribution of oxidative stress to cellular damage, functional impairments and aging. On a per gram basis, myocardial cell mitochondria generate more H_2O_2 compared to other organs (Radi et al., 1991). This was attributable to the high aerobic capacity and

mitochondrial density of cardiomyocytes. Mitochondrial dysfunction and elevated ROS are considered to contributing age-related impairments in cardiovascular function (Radi et al., 1991). Here we investigated the use of a complex dietary supplement designed in part to preserve mitochondrial oxidative capacity that otherwise declines with age. We present measurements of background hydrogen peroxide in whole-heart homogenate and catalase activity in Nr and Tg mouse models across a broad range of ages, as well as data on left venrtricular hypertrophy known to be a key symptom of age-related pathology (Anversa et al., 1990; Olivetti, Melissari, Capasso, & Anversa, 1991). We hypothesized that supplemented Nr and Tg mice would exhibit lower oxidative stress with age compared to untreated mice, either through reduced H_2O_2 and/or the elevation of catalase activity.

The H_2O_2 background remained relatively unchanged in Nr animals with respect to age, regardless of diet ($p = 0.33$, ANCOVA; fig. 12). Rollo (1996), however, found that superoxide radical and lipid peroxides increased with age, and more so in Tg. Untreated Nr animals were unchanged with age ($b = -0.14$, $p = 0.81$, fig 12a), while the supplemented animals showed a marginal age-associated decline in H_2O_2 by 32 months ($b = -0.21$, $p = 0.055$; fig. 12b). In contrast, age-related trends in catalase activity showed significant increases in young (3 months-old) supplemented males (+55%, fig. 14a) compared to age-matched untreated counterparts, with gradual declines by old age ($b = -0.13$, $p = 0.030$). The shorter-lived Tg show a trend for increased hydrogen peroxide background with age in untreated ($b = 0.29$, $p = 0.15$; fig. 12b) and supplemented ($b = 0.38$, $p = 0.52$) diets, consistent with our previous findings that Tg exhibit elevated free radical processes with age (Rollo et al., 1996). Catalase activity in young supplemented

Tg (3 months-old) was 50% higher than age-matched untreated mice, and declined (b = -0.13, p = 0.03) to levels comparable to untreated Nr mice by 18 months of age. The upregulation of catalase activity in young supplemented Nr and Tg mice supports of our hypothesis that the DSP lowers markers of oxidative stress through enhanced antioxidant defenses. The implication of these findings extends to the FRTA as antioxidants offer protection against the age-related accumulation of oxidative damage to cells, organelles and DNA. The increased catalase activity in heart tissue of supplemented mice in younger ages may protect aspects of cellular and mitochondrial functions that are compromised with elevated oxidative stress in older ages.

Catalase protects against high levels of hydrogen peroxide in cardiomyocytes (i.e. lethal sarcolemmal disruption) and is crucial to the maintenance of cardiovascular function {{39 Ward,C.A. 1997}}. Lipid peroxidation, protein damage and ATP loss were observed during hydrogen peroxide-induced oxidative damage {{40 Janero,D.R. 1991; 47 Mak,S. 2001; 43 Seiva,F.R. 2008}}. Interestingly, even a brief "pulse" of hydrogen peroxide was sufficient to trigger cellular disruption in isolated cardiomyocytes {{48 Li,P.F. 1997}}, suggesting that chronically elevated levels of hydrogen peroxide background may not always be required for pathological myocardial oxidative damage. Muscari et al. (1990) found significant age-dependent increases in hydrogen peroxide and lipid peroxides in rat heart was associated with impairments in mitochondrial complex I and III activity, manganese superoxide dismutase (Mn-SOD) and a decreased GSH:GSSG ratio {{49 Muscari,C. 1990}. Furthermore, in adult rat cardiac tissue hydrogen peroxide can cause contractile abnormalities associated with calcium loading in cardiac myocytes

{{38 Josephson,R.A. 1991}}. This was caused by the opening of a pore in the inner-mitochondrial membrane in response to the calcium overload, and can even lead to cell death {{50 Halestrap,A.P. 2010}}.

Signaling by growth hormone (GH) and insulin-like growth factor-1 (IGF-1) are critical for cardiovascular health, as both deficiency and excess increases the risk for cardiovascular pathologies {{43 Seiva,F.R. 2008}}. This was probably due to the direct and indirect (via IGF-1) ability of GH to modulate oxidative metabolism and increase ROS as superoxide and lipid peroxidation were most elevated in the Tg heart and brain {{42 Rollo,C.D. 1996}}. Excess growth hormone secretion in adults (acromegaly) causes pathological hypertrophy of the left ventricle wall, and is strongly linked to cardiac failure {{53 Clemente Gallego,D. 2009; 51 Colao,A. 2011; 55 Fedrizzi,D. 2008; 54 Izzard,A.S. 2009; 52 Toumanidis,S.T. 2011}}. Principally, this is thought to arise from a compensatory response to increased mechanical workload (i.e., pressure and volume) on the terminally differentiated myocytes {{43 Seiva,F.R. 2008}}. The "concentric hypertrophic response" (increased myocyte width and eventually apoptosis) is mediated by coordinated gene expression via IGF-1, IGF receptors, and IGF binding proteins (IGFBPs) in the left ventricle {{51 Colao,A. 2011}}. A GH Tg mouse was recently recognized as a model of acromegaly {{54 Izzard,A.S. 2009}}. Izzard et al. (2009) concluded that the high incidence of cardiovascular mortality was directly linked to hypertrophic cardiomyopathy and not secondary features such as atherosclerosis in these mice {{3 Ingram,D.K. 2000}}. We found that untreated Tg mice exhibited left ventricular hypertrophy compared to supplemented Tg mice, as indicated by gross morphological

measurements ($p = 0.028$, table 2). A similar effect of diet was observed between untreated and supplemented Nr mice ($p = 0.017$), although, we suspect it was more likely attributable to age-associated elevations in oxidative stress {{57 Baumer,A.T. 2000; 56 Seddon,M. 2007}}. Regardless, Aksenov et al. (2010) found that the greatest ROS stress in Tg appears to occur in young mice, followed by dramatic declines in mitochondrial activity in old age (Aksenov et al., 2010). This suggests that some features of age-related pathology may transfer forward from relatively youthful ages.

ROS play an important role in the regulation of tissue energy metabolism and the pathophysiology of cardiovascular function {{44 Bhimaraj,A. 2012; 45 Watanabe,K. 2010; 46 Romuk,E. 2011}}. The relative importance of catalase activity in myocardial tissue compared to other antioxidant enzymes such as glutathione peroxidase (GPx) has been questioned. Although GPx is regarded as the primary scavenger of H_2O_2 in heart, it is also important to consider that H_2O_2 detoxification via GPx oxidizes GSH and this could stress cells or alter signaling systems {{58 Antunes,F. 2002}}. Furthermore, peroxisomal H_2O_2 is generally scavenged by catalase during high oxidative stress when the glutathione system is overwhelmed {{58 Antunes,F. 2002; 41 Radi,R. 1991}}. In fact, Radi et al. (1991) suggested that despite low intracellular concentrations, catalase efficiency is substantially enhanced because it is strategically located near sites where H_2O_2 is generated in heart mitochondria and cytosol {{41 Radi,R. 1991}}. Mouse models overexpressing mitochondrial catalase (liver, kidney, heart and muscle tissue) show improved longevity (~5 months) and reductions in oxidative stress, DNA damage and mitochondrial DNA deletion {{60 Schriner,S.E. 2005}}. Post-mortem heart samples from

acromegalic patients revealed no differences in mRNA and protein of GPx, Mn-SOD and cytosolic SOD in comparison to healthy subjects {{57 Baumer,A.T. 2000}}. Catalase mRNA and protein were unchanged as well; however activity was reduced by 27% indicating post-translational modifications may have caused enzymatic inactivation (Baumer et al., 2000). Baumer et al. (2000) propose that since other scavenging pathways remain unchanged, the decline of catalase activity may have lead to an intracellular redox shift that increased oxidative stress (i.e. hydroxyl radical formation via Fenton reactions) (Baumer et al., 2000).

There is considerable evidence supporting a cardioprotective role for catalase in the maintenance of mitochondrial integrity and intracellular constituents. Our DSP increased mean catalase activity by ~33% in whole-heart homogenate from both Nr and Tg mice. Previous work from our laboratory shows that the DSP increased longevity in Nr and Tg by ~11% and ~28% respectively (Lemon et al., 2005). Rollo et al (1996) found a high correlation between oxidative stress and longevity in heart and brain, particularly in Tg (Rollo et al., 1996). Tg mice represent an ideal model to study the interaction of accelerated aging and acromegaly given the common pathophysiological cause (i.e. GH-axis elevation). Although we cannot differentiate between the specific intracellular locations where catalase activity was enhanced (cytsolic, mitochondrial and/or peroxisomal) our results suggest catalase is a critical antioxidant enzyme in the heart. The increased activity of a key antioxidant enzyme is in accordance with the formulation criteria of the DSP. The results presented here support that the DSP may be a useful intervention for cardiovascular health and function.

4.3.2 Kidney tissue

Kidney pathology is strongly associated with age-relate function (Choksi, Nuss, Boylston, Rabek, & Papaconstantinou, 2007; Kwong & Sohal, 2000) and increased oxidative stress in mice (Galle & Seibold, 2003). This is particularly severe in Tg (Doi et al., 1991). Kidney superoxide radical and lipid peroxidation were elevated in aging mice, particularly Tg (Rollo et al. 1996). Here, we measured H_2O_2 and catalase activity in kidney homogenate from untreated and supplemented Nr and Tg male mice with respect to age. The goal was to understand changes in the balance between oxidant and antioxidants, and to document impacts of our DSP on aging kidney function. Lowering oxidative stress is one of the key targets of the DSP, therefore we hypothesized that this would be reflected in reductions in H_2O_2 and/or elevated catalase activity.

In Nr mice, kidney H_2O_2 showed significant opposite age-related trends with diet ($p = 0.0040$, **fig. 16a**) with untreated Nr showing significant declines ($b = -0.19$, $p = 0.025$) and supplemented Nr significantly increasing ($b = 0.35$, $p = 0.045$). However, the magnitude of overall change was only 2-3% and the effect of diet was not resolved. However, the relationship between H_2O_2 and age illustrated in **fig. 16a** resembles the change in brain mitochondrial complex III and IV with age observed in previous studies using the DSP (**see fig. 21**) (Aksenov et al., 2010, 2011). Although the measurements were taken in different tissues, if a comparable increase in kidney mitochondria activity was achieved in the DSP group, we would expect H_2O_2 to increase significantly as a reflection of electron transport chain activity. In that case the relative output of ROS (assuming a concomitant increase in superoxide dismutase activity) would be lower

compared to rate of ATP synthesis. Effectively, this means that our supplement may elevate mitochondrial function (which is desired) while at the same time maintaining steady H_2O_2 levels (i.e. beneficial for energy production but without the accompanying increase in ROS generation). This would provide benefits associated with the maintenance of physiological functions in the kidney and promote healthy aging.

Catalase activity did not change between untreated and supplemented Nr ($p = 0.29$, **fig. 18a**). Furthermore, in Tg, age-related trends of H_2O_2 remained unchanged across age in untreated ($b = -0.34$, $p = 0.076$) and supplemented ($b = -0.12$, $p = 0.56$) diets ($p = 0.42$, **fig.16b**). Similarly, catalase activity in untreated and supplemented Tg was very similar ($p = 0.68$, **fig. 18a**). Tg are a model of accelerated-aging and exhibit elevated superoxide radicals and lipid peroxidation (Rollo et al., 1996). Others reported that in middle-aged mice over-expressing GH, CuZn-SOD and glutathione peroxidase activity declined with age, but changes in catalase were not assessed (Hauck & Bartke, 2001). Excess GH also correlates with increased circulating IGF-1 and kidney-derived IGF-1 and is implicated in a compensatory renal hypertrophic response, and changes characteristic of diabetes mellitus (Hammerman, 1999). Furthermore, excess GH increases the accumulation of extracellular matrix components in the glomeruli in mice, and contributes to the development of glomerulosclerosis (Doi et al., 1991).

Hydrogen peroxide in the renal cortex and isolated glomeruli increase as a function of age in rats and correlates with increased glomerular damage (Galle & Seibold, 2003; Guidet & Shah, 1989). In addition, the level of renal H_2O_2 is dependent on the balance between SOD and catalase activity, where too much SOD activity (increased

hydrogen peroxide formation) could overwhelm catalase capacity, allowing formation of highly reactive hydroxyl radicals via Fenton reactions (Galle & Seibold, 2003). Catalase is a ubiquitous antioxidant enzyme, however, changes in its activity in relation to various age-related renal pathologies are often inconsistent due to experimental conditions as reviewed by Baylis et al. (1998) (C. Baylis & Corman, 1998). In addition, when considering the neutralization of H_2O_2, it has been suggested that the role of glutathione peroxidase may be more prominent, particularly in renal pathologies such as uremia (Klemm et al., 2001). This was also true in the kidneys of type II diabetic rats, where a time course study found that catalase activity was rapidly compromised but glutathione peroxidase was not (Kakkar, et al., 1997). Furthermore, the roles of excess superoxide anion production or reduced MnSOD activity are also strongly implicated in the pathology of diabetic nephropathy (Mollsten et al., 2007). Interestingly, our findings are consistent with Schriner et al. (2005) who reported no changes in mitochondrial catalase activity within the kidneys of transgenic mice engineered to ubiquitously over-express catalase (Schriner et al., 2005). It seems that the relationship between H_2O_2 and catalase activity is complicated by the influence of other antioxidant enzymes and ROS within the kidney and warrants further investigation. We conclude that our DSP did not alter age-related trends in H_2O_2 or catalase activity in Nr and Tg kidney. Thus, our hypothesis that the DSP would alter ROS or antioxidants was not supported for catalase and H_2O_2 in kidney.

We also monitored 24-hour water consumption patterns in untreated and supplemented Nr mice across ages of 3-30 months (**fig. 4**). Unfortunately we had

insufficient Tg mice available for this assessment. Water consumption was similar at younger ages (3 months-old) in Nr mice. By 24 months untreated mice showed a significant increase in water consumption (+50%, b = 0.22; p = 0.018), but supplemented mice showed no change (b = -0.0076, p = 0.92). Excess fluid intake, particularly in older ages, is a characteristic symptom of polydipsic diabetes insipidus, and strongly implicates declining renal function (P. H. Baylis & Cheetham, 1998). Under normal conditions, plasma osmolality is tightly controlled by vasopressin, a pituitary hormone that regulates water retention through the kidneys (P. H. Baylis & Cheetham, 1998). Age-related impairments in kidney structure and function may reduce the sensitivity of renal osmoreceptors to vasopressin thereby increasing water loss (P. H. Baylis & Cheetham, 1998; Brokaw, 1953). Increased water excretion may dilute urine (polyuria) and lower the osmotic threshold for the onset of thirst sensation and increase hypothalamic vasopressin secretion. In addition, excess thirst is also symptomatic of proteinuria (excess protein excretion in urine), a reliable indicator of end-stage renal failure that develops in part due to oxidative stress in the kidney (Kuusniemi et al., 2005). Although we did not find changes in H_2O_2 or catalase activity, the DSP appears to have protective effects against renal injury as supplemented mice retain osmoregulatory capacity in old age, as indicated by daily water consumption patterns (**fig. 4**). Therefore, it is possible that other indices of oxidative or energetic stress may have changed with age and warrants further study.

4.4 Oxidized:reduced glutathione ratio (GSSG:GSH)

Glutathione (GSH) represents the main cellular antioxidant system and occurs throughout bodily fluids and various tissues. GSH is also critical in redox-sensitive signal transmission that applies to numerous systems (particularly kinase-phosphatase regulated transduction pathways (Droge & Schipper, 2007)). Thus, besides reflecting oxidative conditions, the status of the GSH system can also indicate distortions in important signaling systems that may contribute to aging pathologies. GSH protects tissues from general oxidative stress by scavenging ROS and preventing lipid peroxidation reactions (Y. Zhang et al., 2009). Furthermore, GSH can neutralize hydrogen peroxide to water and lipid hydroperoxides to their respective alcohols, through the action of glutathione peroxidases. As intracellular oxidative stress rises, glutathione peroxidase catalyzes the formation of a disulfide bond between two GSH molecules, resulting in oxidized glutathione (GSSG) (Owen & Butterfield, 2010). Under healthy conditions, up to 98% of the endogenous GSH pool occurs in the reduced form. Therefore, increases in the ratio of GSSG:GSH provides a reliable indicator of oxidative stress and can easily be measured in blood, urine and various tissues. The GSH:GSSG ratio in urine is likely a very sensitive biomarker of whole-body oxidative stress. In the present study, we measured the GSH:GSSG ratio in urine samples from older (≥ 12 months-old) untreated and supplemented Nr male mice, and predicted lower whole-body oxidiative stress (increased GSH:GSSG ratio) in supplemented mice compared to untreated controls. The results were consistent with our hypothesis, as the DSP effectively maintained a higher GSH:GSSG ratio (~41%, $p < 0.001$; **fig. 19**), suggestive of lower whole-body oxidative stress.

Although urine contains μM concentrations of GSH, it remains detectable and quantifiable using commercially available fluorometric assay kits. This also provided the added benefit of a noninvasive measure of oxidative stress, as we wanted to avoid sacrificing old mice that were to be used in behavioral studies.

Urinary markers of various oxidative damage end-products appear to be reliable measures and are widely used as *in vivo* measurements in studies of health and disease (Leeuwenburgh, Hansen, Holloszy, & Heinecke, 1999) (Davies, Fu, Wang, & Dean, 1999; Kadiiska et al., 2005; Leeuwenburgh et al., 1999; Pfeiffer et al., 1999). Halliwell et al. (2004) demonstrated that hydrogen peroxide is readily detectable in human urine samples, but raised cautions regarding storage, stability and reproducibility due intra-individual variability (Halliwell, Long, Yee, Lim, & Kelly, 2004). However, these issues appear to be more limiting in studies involving human subjects where confounding variables are not controlled (i.e. changes in diet and smoking). This issue was also addressed by Yuen and Benzie (2003), who confirm the usefulness of urine hydrogen peroxide as a biomarker of oxidative stress, particularly when disease, therapy and dietary intervention are able to induce large changes in hydrogen peroxide concentration (Yuen & Benzie, 2003).

Extracellular glutathione is used by tissues as a source of intracellular cysteine (glutathione precursor) via membrane bound gamma-glutamyl transpeptidase enzymes (F. Zhang, Lau, & Monks, 2011). Cells release glutathione of both redox states and can be detected in plasma and urine (Hahn, Wendel, & Flohe, 1978). Also, glutathione S-transferase is a ubiquitous enzyme involved in cellular detoxification through conjugation

reactions involving glutathione, and its detection in urine is regarded as a biomarker of renal dysfunction (Walshe, Odejayi, Ng, & Marsh, 2009). Oxidative stress is among the targeted mechanisms of the DSP, and endogenous glutathione represents a prominent antioxidant. Taken together, the DSP appears to enhance the ability of glutathione in protection against whole-body oxidative stress in aged mice, as indicated by the magnitude of change observed in the GSH:GSSG ratio in urine. This result adds to the growing list of benefits observed in supplemented mice and adds to the promise of our DSP in promoting healthy aging. Despite the negative results in kidney, significant alterations in urinary GSH:GSSH ratio strongly support the action of the DSP to reduce oxidative stress in mice.

4.5 Intra-peritoneal glucose tolerance test (IPGTT)

Glucose sensitivity is highly relevant to cellular energy supply and is particularly highlighted in diabetes, kidney function and cardio-muscular functioning (Barr et al., 2007; Chang & Halter, 2003; Fliser et al., 1998; Lindstrom et al., 2003; Lindstrom et al., 2003; Stevic et al., 2007; Tuomilehto et al., 2001). A key mechanism targeted in formulating the DSP was insulin sensitivity, but to date effectiveness had not been assessed.

The ability to tolerate a glucose load can provide valuable insight regarding the status of carbohydrate metabolism. Glucose intolerance associated with age accompanies type 2 diabetes (Chang & Halter, 2003; Stevic et al., 2007). The time required to clear glucose from the blood is indicative of the functional capacity of several physiological

mechanisms involved. It has been widely documented that whole-body glucose tolerance generally declines with age (Basu et al., 2003; Chang & Halter, 2003; Leiter, Premdas, Harrison, & Lipson, 1988; Stevic et al., 2007). This may be attributable to a number of factors including but not limited to: decreased physical activity, impaired insulin secretion and increased adiposity (Chang & Halter, 2003). In the present study we administered a 1 g/kg intra-peritoneal glucose challenge in aged (>12 months-old) untreated and supplemented Nr male mice. We hypothesized that whole-body glucose tolerance would improve in supplemented mice compared to untreated controls, as insulin sensitivity is one of the targeted mechanisms of the supplement. The DSP improved whole-body glucose tolerance as mean AUC was found to be marginally ($p = 0.056$, **fig. 20**) lower in old supplemented Nr, along with an earlier peak in blood glucose that was significantly reduced by 60 minutes ($p < 0.01$) compared with untreated Nr mice.

There are many factors that contribute to the age-related decline in glucose tolerance. For example, decreased physical activity in older ages is associated with impaired insulin sensitivity, and increased risk for type 2 diabetes (N. Owen et al., 2010). This is consistent with our findings of significant age-related declines in physical activity in untreated Nr (**figs. 1-3**). Furthermore, elderly individuals generally exhibit reduced insulin secretion which can contribute to glucose intolerance (Basu et al., 2003). It is also important to consider the role of insulin-independent mechanisms as well, (i.e. uptake by central nervous system) that may account for up to 70% of glucose clearance. Furthermore, impairments in the ability to regulate blood glucose under basal insulin conditions either through increased uptake or decreased production by the liver is an

independent risk factor for type II diabetes (Pacini, Thomaseth, & Ahren, 2001). Studies in rats highlight age-related declines in insulin-stimulated GLUT4 protein content and insulin receptor binding in heart, diaphragm and skeletal muscle samples, contributing to the development of insulin resistance (Wang, Bell-Farrow, Sonntag, & Cefalu, 1997). In a study evaluating the role of diet-induced obesity in several strains of mice, Surwit et al. (1991) conclude that C57BL/6J mice may be predisposed to the development of type II diabetes, suggesting the importance of genetic factors in the regulation of glucose tolerance (Surwit, Seldin, Kuhn, Cochrane, & Feinglos, 1991). This was one of the strains contributing to the hybrid background of our mice. Improved blood glucose tolerance in supplemented mice supports our hypothesis that the DSP can augment aspects of insulin sensitivity, one of its key targeted mechanisms. The ability to maintain insulin sensitivity significantly reduces the risk for a number of age-related pathologies as discussed previously. This reflects physiological stability in important aspects of energy homeostasis and should improve the quality of life and independence in old age.

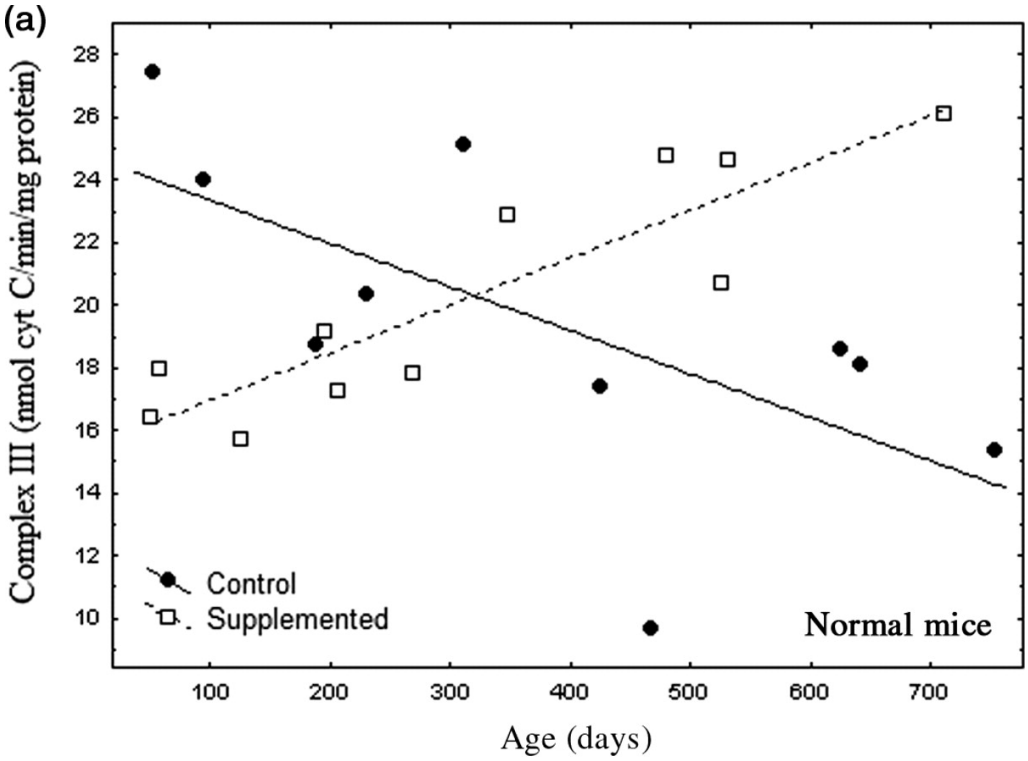
From *(Aksenov et al. 2010)*

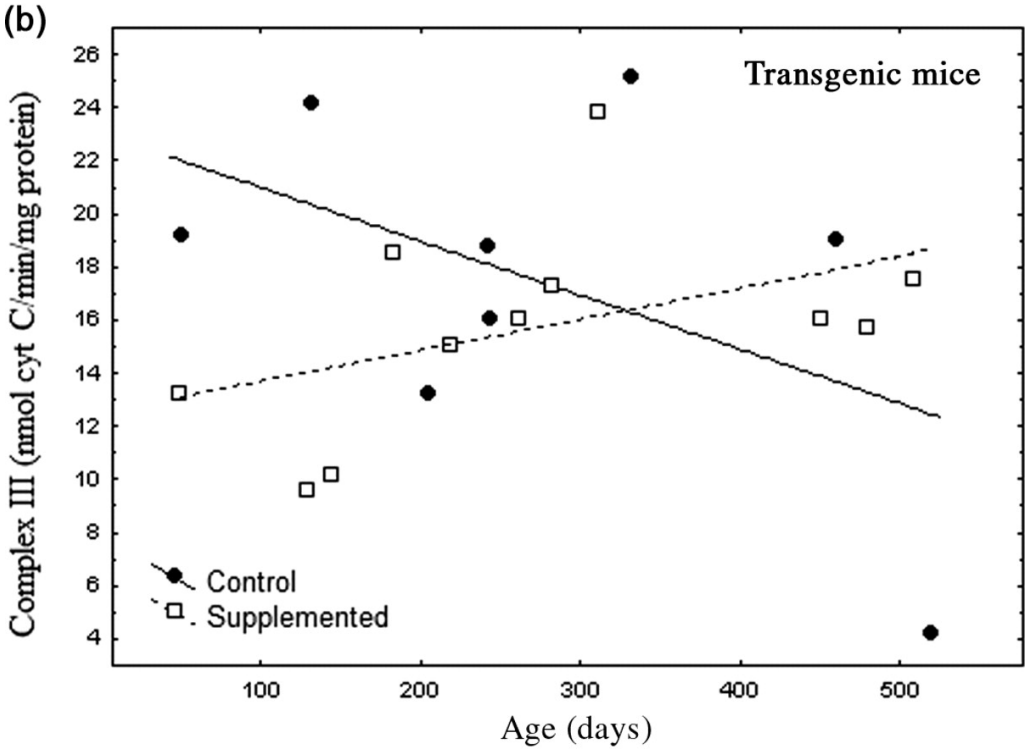
Figure 21. (a) Relationship between age and mitochondrial complex III activity in brains of untreated (n = 10) and supplemented (n = 11) normal mice. Both regressions were significant, and slopes significantly differed, p = 0.0003

Untreated: r = 20.656, linear regression: p = 0.04; complex III activity = 24.735 - 0.014(age)

Supplemented: r = 0.891, linear regression: p = 0.0003; complex III activity = 15.443 + 0.015(age)

(b) Although similar in pattern to normal mice, linear regression models for mitochondrial complex III in supplemented and untreated transgenic (Tg) mice were not significant (p = 0.16 and p = 0.22, respectively). Slopes marginally differed, p = 0.057. Note the apparent depression of complex III activity by the supplement in Tg mice younger than 150 days. Overall, transgenic complex III activity was ~20% lower than in normal mice.


(a)
Complex III (nmol cyt C/min/mg protein)
28
26
24
22
20
18
16
14
12
10
Control
Supplemented
Normal mice
100
200
300
400
500
600
700
Age (days)


(b)
Complex III (nmol cyt C/min/mg protein)
26
24
22
20
18
16
14
12
10
8
6
4
Transgenic mice
Control
Supplemented
100
200
300
400
500
Age (days)

4.6 Conclusions and future directions

My results support the value of carefully designed complex dietary supplements in prolonging functional capacities into old age and its potential use in augmenting youthful exercise capacity. Monitoring age-related changes in physical activity over a 24-hour period is useful in understanding an important component of daily energy expenditure and metabolism. Voluntary physical activity generally declines with age, and our previous findings showed that spontaneous locomotor activity in old supplemented mice is maintained at youthful levels compared to progressive steady declines in untreated controls (Aksenov et al., 2010). I found that total physical activity in untreated mice was lower than that of supplemented mice across all ages. This suggested the relatively novel idea that aspects of youthful function may actually pay forward to functionality in old age. Total activity significantly declined by 17% in untreated mice by 24 months, compared to a non-significant 10% decline in supplemented mice. The changes in activity are largely attributable to declines during the photophase, and parallel findings that mean number of hours resting per day was 40% higher in untreated compared to supplemented mice. Declining physical activity is one of the most reliable biomarkers of aging and for humans, maintaining physical activity into old age improves the quality of life and independence. The impact of the DSP on physical activity has strong implications for both youthful athleticism and geriatric functionality.

Remarkably, the DSP reversed age-related trends in fasting oxygen consumption and RQ in supplemented mice. Untreated animals showed declining oxygen consumption with age and appear to use a relatively greater proportion of fat as a metabolic substrate

fuel source. Conversely, fasting oxygen consumption was ~25% lower in young supplemented mice compared to untreated mice, but significantly increased by 12 months. In a non-fasted state, supplemented mice maintained similar levels of oxygen consumption across age, but untreated mice showed significant declines with age. The resting respiratory exchange ratio was elevated in supplemented mice across all ages compared to untreated mice. Taken together, increased carbohydrate oxidation seems most likely, as more ATP is yielded per mole of oxygen compared to protein or lipids (Hochachka et al., 1991). This suggests important alterations in the metabolic costs for locomotion. Reduced energetic costs could both support athleticism and contribute to greater geriatric functionality since movement is associated with increased costs with age (Malatesta et al., 2003). DSP-treated mice appear more metabolically efficient in old age consistent in keeping with our previous reports of increased brain mitochondrial complex III and IV activity (Aksenov et al. 2010, 2011). Future experiments should assess mitochondrial function in skeletal muscle, as well as changes in body composition (i.e. changes in fat-free mass) that may provide insight into the progression of energy efficiency and sarcopenia in old age. The DSP may be useful as an ergogenic aid in exercise performance for young and old subjects which presents an exciting avenue of future research.

We measured H_2O_2 background and catalase activity in heart and kidney from Nr and Tg mice. In the heart, H_2O_2 background was similar across age in untreated and supplemented Nr and Tg mice, but mean H_2O_2 background was higher in Tg mice compared to Nr mice. However, the DSP elevated mean catalase activity by ~30% in both

genotypes compared to untreated controls. Catalase upregulation suggests increased protection against oxidative damage. Given that H_2O_2 did not change, this suggests that the DSP may actually have increased basal H_2O_2 generation and catalase elevation was offsetting. In addition, cardiac hypertrophy often develops as a compensatory response to declining cardiac function, and oxidative stress is among the etiologic factors. We measured left ventricular wall thickness, an indicator of cardiac hypertrophy, and found it to be significantly lower in Nr and Tg supplemented mice, compared to untreated controls. This suggests that the DSP may be protecting heart tissue from mechanical/ROS stress that can compromise physiological function with age, particularly in Tg (a model of acromegaly) (Izzard et al., 2009). Future experiments should characterize other indices of oxidative stress (i.e. superoxide anion production, CuZn-SOD and Mn-SOD activity and especially known radicals known to be particularly damaging). In particular, perceived weaknesses with the free radical theory may well arise as free radicals like superoxide and hydrogen peroxide may be relatively harmless and are actually required for normal redox signaling. Oxidative stress relevant to aging may be associated more with more damaging free radicals such as hydroxyl radical, 4-hydroxynonenal and peroxynitrite.

In the kidney, the DSP reversed age-related trends of H_2O_2 of Nr mice, with untreated mice showing significant declines with age, and supplemented mice showing significant increases. In Tg mice, age-related trends of H_2O_2 levels between diets were very similar to Nr and generally did not change with age. Catalase activity remained unchanged in both untreated and supplemented groups in Nr and Tg mice. Although these measures in the kidney were not significantly altered by the DSP, they represent a few of

many possible biomarkers of oxidative stress that could affect renal function with age. Previous results (Rollo et al. 1996) strongly suggest that other ROS processes in kidney indeed respond to both diets and mouse strains (particularly Tg). Furthermore, the polydipsia in untreated mice reflected a 50% increase in daily water consumption by 24 months, compared to no increase in supplemented mice. This is often characteristic of diabetes insipidus or end-stage renal failure (Baylis & Cheetham, 1998), and suggests our DSP may conserve aspects of renal function in old age, presenting promising avenues of future study.

The ratio of reduced (GSH) to oxidized (GSSG) glutathione is sensitive to changes in oxidative status, as the intracellular glutathione pool is generally maintained in the reduced state (Owen & Butterfield, 2010). Our measurements of this ratio in urine from older (≥12 months-old) untreated and supplemented Nr male mice provide an indicator of whole-body oxidative stress. We found a 43% increase in the GSH:GSSG ratio in urine samples from supplemented mice compared to untreated mice. This result suggests that our DSP strongly lowers whole-body oxidative stress, and should be followed up with tissue-specific and plasma measurements of the GSH:GSSG ratio.

One of the five targeted mechanisms of aging for the DSP was insulin sensitivity. To assess whether the supplement was achieving this goal whole-body glucose tolerance was assessed in older (≥12 months-old) untreated and supplemented male mice. Although mean area under the curve (AUC) was marginally lower in the supplemented group, blood glucose values peaked earlier and were significantly lower by 60 minutes compared to untreated mice ($p < 0.01$, **fig. 20**). The regulation of blood glucose is complex and

changes with age. Our results show promise in the ability of our DSP to improve glucose tolerance and should be examined in future investigations. This may include an intra-peritoneal glucose tolerance test in younger (<12 months-old) mice for comparison, and associated changes in insulin.

As the human population develops an increased proportion of individuals >60 years of age, there is an urgent need to develop practical solutions to promote health and functional longevity. Malnutrition generally accompanies aging, and is problematic for elderly individuals who often find it difficult maintain a balanced diet. This may increase the potential importance of dietary supplementation at such ages. Conveniently, ingredients in our DSP are easily available in health food stores that are accessible to a majority of the population. The results presented here add to the growing promise that complex dietary supplements can offset key mechanisms involved in aging and perhaps improve the quality of life in elderly humans.

REFERENCES

Aksenov, V., Long, J., Liu, J., Szechtman, H., Khanna, P., Matravadia, S., et al. (2011). A complex dietary supplement augments spatial learning, brain mass, and mitochondrial electron transport chain activity in aging mice. *Age (Dordrecht, Netherlands),* doi:10.1007/s11357-011-9325-2

Aksenov, V., Long, J., Lokuge, S., Foster, J. A., Liu, J., & Rollo, C. D. (2010). Dietary amelioration of locomotor, neurotransmitter and mitochondrial aging. *Experimental Biology and Medicine (Maywood, N.J.), 235*(1), 66-76. doi:10.1258/ebm.2009.009219

Anversa, P., Palackal, T., Sonnenblick, E. H., Olivetti, G., Meggs, L. G., & Capasso, J. M. (1990). Myocyte cell loss and myocyte cellular hyperplasia in the hypertrophied aging rat heart. *Circulation Research, 67*(4), 871-885.

Banks, R., Speakman, J. R., & Selman, C. (2010). Vitamin E supplementation and mammalian lifespan. *Molecular Nutrition & Food Research, 54*(5), 719-725. doi:10.1002/mnfr.200900382

Barr, E. L., Zimmet, P. Z., Welborn, T. A., Jolley, D., Magliano, D. J., Dunstan, D. W., et al. (2007). Risk of cardiovascular and all-cause mortality in individuals with diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance: The Australian diabetes, obesity, and lifestyle study (AusDiab). *Circulation, 116*(2), 151-157. doi:10.1161/CIRCULATIONAHA.106.685628

Bartke, A. (2009). The somatotropic axis and aging: Mechanisms and persistent questions about practical implications. *Experimental Gerontology, 44*(6-7), 372-374. doi:10.1016/j.exger.2009.04.001

Bartke, A., Brown-Borg, H., Mattison, J., Kinney, B., Hauck, S., & Wright, C. (2001). Prolonged longevity of hypopituitary dwarf mice. *Experimental Gerontology, 36*(1), 21-28.

Bass, T. M., Weinkove, D., Houthoofd, K., Gems, D., & Partridge, L. (2007). Effects of resveratrol on lifespan in drosophila melanogaster and caenorhabditis elegans. *Mechanisms of Ageing and Development, 128*(10), 546-552. doi:10.1016/j.mad.2007.07.007

Basu, R., Breda, E., Oberg, A. L., Powell, C. C., Dalla Man, C., Basu, A., et al. (2003). Mechanisms of the age-associated deterioration in glucose tolerance: Contribution of alterations in insulin secretion, action, and clearance. *Diabetes, 52*(7), 1738-1748.

Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., et al. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. *Nature, 444*(7117), 337-342. doi:10.1038/nature05354

Baylis, C., & Corman, B. (1998). The aging kidney: Insights from experimental studies. *Journal of the American Society of Nephrology : JASN, 9*(4), 699-709.

Baylis, P. H., & Cheetham, T. (1998). Diabetes insipidus. *Archives of Disease in Childhood, 79*(1), 84-89.

Bezlepkin, V. G., Sirota, N. P., & Gaziev, A. I. (1996). The prolongation of survival in mice by dietary antioxidants depends on their age by the start of feeding this diet. *Mechanisms of Ageing and Development, 92*(2-3), 227-234.

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

Bosy-Westphal, A., Eichhorn, C., Kutzner, D., Illner, K., Heller, M., & Muller, M. J. (2003). The age-related decline in resting energy expenditure in humans is due to the loss of fat-free mass and to alterations in its metabolically active components. *The Journal of Nutrition, 133*(7), 2356-2362.

Brand, M. D., Chien, L. F., Ainscow, E. K., Rolfe, D. F., & Porter, R. K. (1994). The causes and functions of mitochondrial proton leak. *Biochimica Et Biophysica Acta, 1187*(2), 132-139.

Brokaw, A. (1953). Renal hypertrophy and polydipsia in potassium-deficient rats. *The American Journal of Physiology, 172*(2), 333-346.

Brown-Borg, H. M. (2009). Hormonal control of aging in rodents: The somatotropic axis. *Molecular and Cellular Endocrinology, 299*(1), 64-71. doi:10.1016/j.mce.2008.07.001

Brown-Borg, H. M., Borg, K. E., Meliska, C. J., & Bartke, A. (1996). Dwarf mice and the ageing process. *Nature, 384*(6604), 33. doi:10.1038/384033a0

Canto, C., & Auwerx, J. (2011). Calorie restriction: Is AMPK a key sensor and effector? *Physiology (Bethesda, Md.), 26*(4), 214-224. doi:10.1152/physiol.00010.2011

Carter, C. S., Ramsey, M. M., & Sonntag, W. E. (2002). A critical analysis of the role of growth hormone and IGF-1 in aging and lifespan. *Trends in Genetics : TIG, 18*(6), 295-301.

Caspersen, C.J., Merritt, R.K, Stephens T. (1994). International physical activity patterns: A methodological perspective international physical activity patterns: A methodological perspective. (pp. 73 - 110)

Castello, L., Maina, M., Testa, G., Cavallini, G., Biasi, F., Donati, A., et al. (2011). Alternate-day fasting reverses the age-associated hypertrophy phenotype in rat heart by influencing the ERK and PI3K signaling pathways. *Mechanisms of Ageing and Development, 132*(6-7), 305-314. doi:10.1016/j.mad.2011.06.006

Chang, A. M., & Halter, J. B. (2003). Aging and insulin secretion. *American Journal of Physiology.Endocrinology and Metabolism, 284*(1), E7-12. doi:10.1152/ajpendo.00366.2002

Chaudhry, A. M., Marsh-Rollo, S. E., Aksenov, V., Rollo, C. D., & Szechtman, H. (2008). Modifier selection by transgenes: The case of growth hormone transgenesis and hyperactive circling mice. *Evolutionary Biology, 35*(4), 267.

Chen, X., Mele, J., Giese, H., Van Remmen, H., Dolle, M. E., Steinhelper, M., et al. (2003). A strategy for the ubiquitous overexpression of human catalase and CuZn superoxide dismutase genes in transgenic mice. *Mechanisms of Ageing and Development, 124*(2), 219-227.

Choksi, K. B., Nuss, J. E., Boylston, W. H., Rabek, J. P., & Papaconstantinou, J. (2007). Age-related increases in oxidatively damaged proteins of mouse kidney mitochondrial electron transport chain complexes. *Free Radical Biology & Medicine, 43*(10), 1423-1438. doi:10.1016/j.freeradbiomed.2007.07.027

Conley, K. E., Jubrias, S. A., Amara, C. E., & Marcinek, D. J. (2007). Mitochondrial dysfunction: Impact on exercise performance and cellular aging. *Exercise and Sport Sciences Reviews, 35*(2), 43-49. doi:10.1249/JES.0b013e31803e88e9

Cooper, J. M., Mann, V. M., & Schapira, A. H. (1992). Analyses of mitochondrial respiratory chain function and mitochondrial DNA deletion in human skeletal muscle: Effect of ageing. *Journal of the Neurological Sciences, 113*(1), 91-98.

Coschigano, K. T., Holland, A. N., Riders, M. E., List, E. O., Flyvbjerg, A., & Kopchick, J. J. (2003). Deletion, but not antagonism, of the mouse growth hormone receptor results in severely decreased body weights, insulin, and insulin-like growth factor I levels and increased life span. *Endocrinology, 144*(9), 3799-3810.

Cousin, K. M., Uretsky, N. J., & Gerald, M. C. (1985). Locomotor response of nialamide pretreated old rats to intraaccumbens dopamine. *Pharmacology, Biochemistry, and Behavior, 22*(3), 461-468.

Csiszar, A., Labinskyy, N., Perez, V., Recchia, F. A., Podlutsky, A., Mukhopadhyay, P., et al. (2008). Endothelial function and vascular oxidative stress in long-lived GH/IGF-deficient ames dwarf mice. *American Journal of Physiology.Heart and Circulatory Physiology, 295*(5), H1882-94. doi:10.1152/ajpheart.412.2008

Cutler, R. G. (1985). Peroxide-producing potential of tissues: Inverse correlation with longevity of mammalian species. *Proceedings of the National Academy of Sciences of the United States of America, 82*(14), 4798-4802.

Davies, M. J., Fu, S., Wang, H., & Dean, R. T. (1999). Stable markers of oxidant damage to proteins and their application in the study of human disease. *Free Radical Biology & Medicine, 27*(11-12), 1151-1163.

Desai, V. G., Weindruch, R., Hart, R. W., & Feuers, R. J. (1996). Influences of age and dietary restriction on gastrocnemius electron transport system activities in mice.

Archives of Biochemistry and Biophysics, 333(1), 145-151. doi:10.1006/abbi.1996.0375

Doi, T., Striker, L. J., Kimata, K., Peten, E. P., Yamada, Y., & Striker, G. E. (1991). Glomerulosclerosis in mice transgenic for growth hormone. increased mesangial extracellular matrix is correlated with kidney mRNA levels. *The Journal of Experimental Medicine, 173*(5), 1287-1290.

Droge, W., & Schipper, H. M. (2007). Oxidative stress and aberrant signaling in aging and cognitive decline. *Aging Cell, 6*(3), 361-370. doi:10.1111/j.1474-9726.2007.00294.x

Edgar, D., & Trifunovic, A. (2009). The mtDNA mutator mouse: Dissecting mitochondrial involvement in aging. *Aging, 1*(12), 1028-1032.

Fliser, D., Pacini, G., Engelleiter, R., Kautzky-Willer, A., Prager, R., Franek, E., et al. (1998). Insulin resistance and hyperinsulinemia are already present in patients with incipient renal disease. *Kidney International, 53*(5), 1343-1347. doi:10.1046/j.1523-1755.1998.00898.x

Flurkey, K., Papaconstantinou, J., Miller, R. A., & Harrison, D. E. (2001). Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. *Proceedings of the National Academy of Sciences of the United States of America, 98*(12), 6736-6741. doi:10.1073/pnas.111158898

Frisard, M., & Ravussin, E. (2006). Energy metabolism and oxidative stress: Impact on the metabolic syndrome and the aging process. *Endocrine, 29*(1), 27-32. doi:10.1385/ENDO:29:1:27

Galle, J., & Seibold, S. (2003). Has the time come to use antioxidant therapy in uraemic patients? *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association, 18*(8), 1452-1455.

Guidet, B. R., & Shah, S. V. (1989). In vivo generation of hydrogen peroxide by rat kidney cortex and glomeruli. *The American Journal of Physiology, 256*(1 Pt 2), F158-64.

Hagen, T. M., Ingersoll, R. T., Wehr, C. M., Lykkesfeldt, J., Vinarsky, V., Bartholomew, J. C., et al. (1998). Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. *Proceedings of the National Academy of Sciences of the United States of America, 95*(16), 9562-9566.

Hagen, T. M., Liu, J., Lykkesfeldt, J., Wehr, C. M., Ingersoll, R. T., Vinarsky, V., et al. (2002). Feeding acetyl-L-carnitine and lipoic acid to old rats significantly improves metabolic function while decreasing oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America, 99*(4), 1870-1875. doi:10.1073/pnas.261708898

Hahn, R., Wendel, A., & Flohe, L. (1978). The fate of extracellular glutathione in the rat. *Biochimica Et Biophysica Acta, 539*(3), 324-337.

Halliwell, B., Long, L. H., Yee, T. P., Lim, S., & Kelly, R. (2004). Establishing biomarkers of oxidative stress: The measurement of hydrogen peroxide in human urine. *Current Medicinal Chemistry, 11*(9), 1085-1092.

Hammerman, M. R. (1999). The growth hormone-insulin-like growth factor axis in kidney re-revisited. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association, 14*(8), 1853-1860.

Haramizu, S., Ota, N., Hase, T., & Murase, T. (2011). Aging-associated changes in physical performance and energy metabolism in the senescence-accelerated mouse. *The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences, 66*(6), 646-655. doi:10.1093/gerona/glr037

HARMAN, D. (1956). Aging: A theory based on free radical and radiation chemistry. *Journal of Gerontology, 11*(3), 298-300.

Hauck, S. J., & Bartke, A. (2001). Free radical defenses in the liver and kidney of human growth hormone transgenic mice: Possible mechanisms of early mortality. *The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences, 56*(4), B153-62.

Hochachka, P. W., Stanley, C., Matheson, G. O., McKenzie, D. C., Allen, P. S., & Parkhouse, W. S. (1991). Metabolic and work efficiencies during exercise in andean natives. *Journal of Applied Physiology (Bethesda, Md.: 1985), 70*(4), 1720-1730.

Huang, T. T., Carlson, E. J., Gillespie, A. M., Shi, Y., & Epstein, C. J. (2000). Ubiquitous overexpression of CuZn superoxide dismutase does not extend life span in mice. *The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences, 55*(1), B5-9.

Ingram, D. K. (2000). Age-related decline in physical activity: Generalization to nonhumans. *Medicine and Science in Sports and Exercise, 32*(9), 1623-1629.

Izzard, A. S., Emerson, M., Prehar, S., Neyses, L., Trainer, P., List, E. O., et al. (2009). The cardiovascular phenotype of a mouse model of acromegaly. *Growth Hormone & IGF Research : Official Journal of the Growth Hormone Research Society and the International IGF Research Society, 19*(5), 413-419. doi:10.1016/j.ghir.2008.12.006

Jang, Y. C., Perez, V. I., Song, W., Lustgarten, M. S., Salmon, A. B., Mele, J., et al. (2009). Overexpression of mn superoxide dismutase does not increase life span in mice. *The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences, 64*(11), 1114-1125. doi:10.1093/gerona/glp100

Kadiiska, M. B., Gladen, B. C., Baird, D. D., Germolec, D., Graham, L. B., Parker, C. E., et al. (2005). Biomarkers of oxidative stress study II: Are oxidation products of

lipids, proteins, and DNA markers of CCl4 poisoning? *Free Radical Biology & Medicine, 38*(6), 698-710. doi:10.1016/j.freeradbiomed.2004.09.017

Kakkar, R., Mantha, S. V., Radhi, J., Prasad, K., & Kalra, J. (1997). Antioxidant defense system in diabetic kidney: A time course study. *Life Sciences, 60*(9), 667-679.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., & Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. *Nature, 366*(6454), 461-464. doi:10.1038/366461a0

Klemm, A., Voigt, C., Friedrich, M., Funfstuck, R., Sperschneider, H., Jager, E. G., et al. (2001). Determination of erythrocyte antioxidant capacity in haemodialysis patients using electron paramagnetic resonance. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association, 16*(11), 2166-2171.

Krause, K. E., McIntosh, E. I., & Vallis, L. A. (2011). Sarcopenia and predictors of the fat free mass index in community-dwelling and assisted-living older men and women. *Gait & Posture,* doi:10.1016/j.gaitpost.2011.09.003

Ku, H. H., Brunk, U. T., & Sohal, R. S. (1993). Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Radical Biology & Medicine, 15*(6), 621-627.

Kuusniemi, A. M., Lapatto, R., Holmberg, C., Karikoski, R., Rapola, J., & Jalanko, H. (2005). Kidneys with heavy proteinuria show fibrosis, inflammation, and oxidative stress, but no tubular phenotypic change. *Kidney International, 68*(1), 121-132. doi:10.1111/j.1523-1755.2005.00386.x

Kwong, L. K., & Sohal, R. S. (2000). Age-related changes in activities of mitochondrial electron transport complexes in various tissues of the mouse. *Archives of Biochemistry and Biophysics, 373*(1), 16-22. doi:10.1006/abbi.1999.1495

Kyle, U. G., Genton, L., Hans, D., Karsegard, L., Slosman, D. O., & Pichard, C. (2001). Age-related differences in fat-free mass, skeletal muscle, body cell mass and fat mass between 18 and 94 years. *European Journal of Clinical Nutrition, 55*(8), 663-672. doi:10.1038/sj.ejcn.1601198

Lapointe, J., & Hekimi, S. (2010). When a theory of aging ages badly. *Cellular and Molecular Life Sciences : CMLS, 67*(1), 1-8. doi:10.1007/s00018-009-0138-8

Le Bourg, E. (1987). The rate of living theory. Spontaneous locomotor activity, aging and longevity in *Drosophila melanogaster*. *Experimental Gerontology, 22*(5), 359-369.

Leeuwenburgh, C., Hansen, P. A., Holloszy, J. O., & Heinecke, J. W. (1999). Oxidized amino acids in the urine of aging rats: Potential markers for assessing oxidative stress in vivo. *The American Journal of Physiology, 276*(1 Pt 2), R128-35.

Leiter, E. H., Premdas, F., Harrison, D. E., & Lipson, L. G. (1988). Aging and glucose homeostasis in C57BL/6J male mice. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 2*(12), 2807-2811.

Lemon, J. A., Boreham, D. R., & Rollo, C. D. (2005). A complex dietary supplement extends longevity of mice. *The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences, 60*(3), 275-279.

Lemon, J. A., Rollo, C. D., & Boreham, D. R. (2008). Elevated DNA damage in a mouse model of oxidative stress: Impacts of ionizing radiation and a protective dietary supplement. *Mutagenesis, 23*(6), 473-482. doi:10.1093/mutage/gen036

Levine, J. A., Eberhardt, N. L., & Jensen, M. D. (1999). Role of nonexercise activity thermogenesis in resistance to fat gain in humans. *Science (New York, N.Y.), 283*(5399), 212-214.

Li, S., Crenshaw, E. B.,3rd, Rawson, E. J., Simmons, D. M., Swanson, L. W., & Rosenfeld, M. G. (1990). Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature, 347*(6293), 528-533. doi:10.1038/347528a0

Lindstrom, J., Eriksson, J. G., Valle, T. T., Aunola, S., Cepaitis, Z., Hakumaki, M., et al. (2003). Prevention of diabetes mellitus in subjects with impaired glucose tolerance in the finnish diabetes prevention study: Results from a randomized clinical trial. *Journal of the American Society of Nephrology : JASN, 14*(7 Suppl 2), S108-13.

Lipman, R. D., Bronson, R. T., Wu, D., Smith, D. E., Prior, R., Cao, G., et al. (1998). Disease incidence and longevity are unaltered by dietary antioxidant supplementation initiated during middle age in C57BL/6 mice. *Mechanisms of Ageing and Development, 103*(3), 269-284.

Liu, J., Head, E., Kuratsune, H., Cotman, C. W., & Ames, B. N. (2004). Comparison of the effects of L-carnitine and acetyl-L-carnitine on carnitine levels, ambulatory activity, and oxidative stress biomarkers in the brain of old rats. *Annals of the New York Academy of Sciences, 1033*, 117-131. doi:10.1196/annals.1320.011

Longo, K. A., Charoenthongtrakul, S., Giuliana, D. J., Govek, E. K., McDonagh, T., Distefano, P. S., et al. (2010). The 24-hour respiratory quotient predicts energy intake and changes in body mass. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 298*(3), R747-54. doi:10.1152/ajpregu.00476.2009

Malatesta, D., Simar, D., Dauvilliers, Y., Candau, R., Borrani, F., Prefaut, C., et al. (2003). Energy cost of walking and gait instability in healthy 65- and 80-yr-olds. *Journal of Applied Physiology (Bethesda, Md.: 1985), 95*(6), 2248-2256. doi:10.1152/japplphysiol.01106.2002

Manini, T. M. (2010). Energy expenditure and aging. *Ageing Research Reviews, 9*(1), 1-11. doi:10.1016/j.arr.2009.08.002

McCue, M. D. (2010). Starvation physiology: Reviewing the different strategies animals use to survive a common challenge. *Comparative Biochemistry and Physiology.Part A, Molecular & Integrative Physiology, 156*(1), 1-18. doi:10.1016/j.cbpa.2010.01.002

Mollsten, A., Marklund, S. L., Wessman, M., Svensson, M., Forsblom, C., Parkkonen, M., et al. (2007). A functional polymorphism in the manganese superoxide dismutase gene and diabetic nephropathy. *Diabetes, 56*(1), 265-269. doi:10.2337/db06-0698

Mookerjee, S. A., Divakaruni, A. S., Jastroch, M., & Brand, M. D. (2010). Mitochondrial uncoupling and lifespan. *Mechanisms of Ageing and Development, 131*(7-8), 463-472. doi:10.1016/j.mad.2010.03.010

Muller, F. L., Song, W., Liu, Y., Chaudhuri, A., Pieke-Dahl, S., Strong, R., et al. (2006). Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. *Free Radical Biology & Medicine, 40*(11), 1993-2004. doi:10.1016/j.freeradbiomed.2006.01.036

Murakami, S. (2006). Stress resistance in long-lived mouse models. *Experimental Gerontology, 41*(10), 1014-1019. doi:10.1016/j.exger.2006.06.061

Olivetti, G., Melissari, M., Capasso, J. M., & Anversa, P. (1991). Cardiomyopathy of the aging human heart. myocyte loss and reactive cellular hypertrophy. *Circulation Research, 68*(6), 1560-1568.

Owen, J. B., & Butterfield, D. A. (2010). Measurement of oxidized/reduced glutathione ratio. *Methods in Molecular Biology (Clifton, N.J.), 648*, 269-277. doi:10.1007/978-1-60761-756-3_18

Owen, N., Healy, G. N., Matthews, C. E., & Dunstan, D. W. (2010). Too much sitting: The population health science of sedentary behavior. *Exercise and Sport Sciences Reviews, 38*(3), 105-113. doi:10.1097/JES.0b013e3181e373a2

Pacini, G., Thomaseth, K., & Ahren, B. (2001). Contribution to glucose tolerance of insulin-independent vs. insulin-dependent mechanisms in mice. *American Journal of Physiology.Endocrinology and Metabolism, 281*(4), E693-703.

Papaconstantinou, J. (2009). Insulin/IGF-1 and ROS signaling pathway cross-talk in aging and longevity determination. *Molecular and Cellular Endocrinology, 299*(1), 89-100. doi:10.1016/j.mce.2008.11.025

Pearl, R. (1928). *The rate of living*

Peoples, G. E., McLennan, P. L., Howe, P. R., & Groeller, H. (2008). Fish oil reduces heart rate and oxygen consumption during exercise. *Journal of Cardiovascular Pharmacology, 52*(6), 540-547. doi:10.1097/FJC.0b013e3181911913

Perez, V. I., Van Remmen, H., Bokov, A., Epstein, C. J., Vijg, J., & Richardson, A. (2009). The overexpression of major antioxidant enzymes does not extend the lifespan of mice. *Aging Cell, 8*(1), 73-75. doi:10.1111/j.1474-9726.2008.00449.x

Petersen, K. F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D. L., et al. (2003). Mitochondrial dysfunction in the elderly: Possible role in insulin resistance. *Science (New York, N.Y.), 300*(5622), 1140-1142. doi:10.1126/science.1082889

Pfeiffer, J. M., Askew, E. W., Roberts, D. E., Wood, S. M., Benson, J. E., Johnson, S. C., et al. (1999). Effect of antioxidant supplementation on urine and blood markers of oxidative stress during extended moderate-altitude training. *Wilderness & Environmental Medicine, 10*(2), 66-74.

Piers, L. S., Soares, M. J., McCormack, L. M., & O'Dea, K. (1998). Is there evidence for an age-related reduction in metabolic rate? *Journal of Applied Physiology (Bethesda, Md.: 1985), 85*(6), 2196-2204.

Radi, R., Turrens, J. F., Chang, L. Y., Bush, K. M., Crapo, J. D., & Freeman, B. A. (1991). Detection of catalase in rat heart mitochondria. *The Journal of Biological Chemistry, 266*(32), 22028-22034.

Randle, P. J. (1995). Metabolic fuel selection: General integration at the whole-body level. *The Proceedings of the Nutrition Society, 54*(1), 317-327.

Ravussin, E., Lillioja, S., Anderson, T. E., Christin, L., & Bogardus, C. (1986). Determinants of 24-hour energy expenditure in man. methods and results using a respiratory chamber. *The Journal of Clinical Investigation, 78*(6), 1568-1578. doi:10.1172/JCI112749

Ravussin, E., & Swinburn, B. A. (1993). Metabolic predictors of obesity: Cross-sectional versus longitudinal data. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity, 17 Suppl 3*, S28-31; discussion S41-2.

Rollo, C. D. (2002). Growth negatively impacts the life span of mammals. *Evolution & Development, 4*(1), 55-61.

Rollo, C. D. (2010). Aging and the mammalian regulatory triumvirate. *Aging and Disease, 1*(2), 105.

Rollo, C. D., Carlson, J., & Sawada, M. (1996). Accelerated aging of giant transgenic mice is associated with elevated free radical processes. *Canadian Journal of Zoology, 74*(4), 606.

Rollo, C. D., Kajiura, L. J., Wylie, B., & D'Souza, S. (1999). The growth hormone axis, feeding, and central allocative regulation: Lessons from giant transgenic growth hormone mice. *Canadian Journal of Zoology, 77*, 1861.

Romanick, M. A., Rakoczy, S. G., & Brown-Borg, H. M. (2004). Long-lived ames dwarf mouse exhibits increased antioxidant defense in skeletal muscle. *Mechanisms of Ageing and Development, 125*(4), 269-281. doi:10.1016/j.mad.2004.02.001

Roth, G. S., & Joseph, J. A. (1994). Cellular and molecular mechanisms of impaired dopaminergic function during aging. *Annals of the New York Academy of Sciences, 719*, 129-135.

Sallis, J. F. (1999). Age-related decline in physical activity: A synthesis of human and animal studies. *Medicine & Science in Sports & Exercise, 0195-9131*(00), 1598.

Salmon, A. B., Richardson, A., & Perez, V. I. (2010). Update on the oxidative stress theory of aging: Does oxidative stress play a role in aging or healthy aging? *Free Radical Biology & Medicine, 48*(5), 642-655. doi:10.1016/j.freeradbiomed.2009.12.015

Schriner, S. E., Linford, N. J., Martin, G. M., Treuting, P., Ogburn, C. E., Emond, M., et al. (2005). Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science (New York, N.Y.), 308*(5730), 1909-1911. doi:10.1126/science.1106653

Selman, C., Lingard, S., Choudhury, A. I., Batterham, R. L., Claret, M., Clements, M., et al. (2008). Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 22*(3), 807-818. doi:10.1096/fj.07-9261com

Selman, C., McLaren, J. S., Meyer, C., Duncan, J. S., Redman, P., Collins, A. R., et al. (2006). Life-long vitamin C supplementation in combination with cold exposure

does not affect oxidative damage or lifespan in mice, but decreases expression of antioxidant protection genes. *Mechanisms of Ageing and Development, 127*(12), 897-904. doi:10.1016/j.mad.2006.09.008

Sentman, M. L., Granstrom, M., Jakobson, H., Reaume, A., Basu, S., & Marklund, S. L. (2006). Phenotypes of mice lacking extracellular superoxide dismutase and copper- and zinc-containing superoxide dismutase. *The Journal of Biological Chemistry, 281*(11), 6904-6909. doi:10.1074/jbc.M510764200

Sohal, R. S., Kamzalov, S., Sumien, N., Ferguson, M., Rebrin, I., Heinrich, K. R., et al. (2006). Effect of coenzyme Q10 intake on endogenous coenzyme Q content, mitochondrial electron transport chain, antioxidative defenses, and life span of mice. *Free Radical Biology & Medicine, 40*(3), 480-487. doi:10.1016/j.freeradbiomed.2005.08.037

Sohal, R. S., & Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. *Science (New York, N.Y.), 273*(5271), 59-63.

Speakman, J. R., Talbot, D. A., Selman, C., Snart, S., McLaren, J. S., Redman, P., et al. (2004). Uncoupled and surviving: Individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell, 3*(3), 87-95. doi:10.1111/j.1474-9728.2004.00097.x

Spong, A., & Bartke, A. (2010). Hormonal influences on aging and lifespan. *The Comparative Biology of Aging,* , 43-68.

Stevic, R., Zivkovic, T. B., Erceg, P., Milosevic, D., Despotovic, N., & Davidovic, M. (2007). Oral glucose tolerance test in the assessment of glucose-tolerance in the elderly people. *Age and Ageing, 36*(4), 459-462. doi:10.1093/ageing/afm076

Sun, M., Qian, F., Shen, W., Tian, C., Hao, J., Sun, L., et al. (2011). Mitochondrial nutrients stimulate performance and mitochondrial biogenesis in exhaustively exercised rats. *Scandinavian Journal of Medicine & Science in Sports,* doi:10.1111/j.1600-0838.2011.01314.x; 10.1111/j.1600-0838.2011.01314.x

Surwit, R. S., Seldin, M. F., Kuhn, C. M., Cochrane, C., & Feinglos, M. N. (1991). Control of expression of insulin resistance and hyperglycemia by different genetic factors in diabetic C57BL/6J mice. *Diabetes, 40*(1), 82-87.

Suzuki, Y. J., Forman, H. J., & Sevanian, A. (1997). Oxidants as stimulators of signal transduction. *Free Radical Biology & Medicine, 22*(1-2), 269-285.

Tatar, M., Kopelman, A., Epstein, D., Tu, M. P., Yin, C. M., & Garofalo, R. S. (2001). A mutant drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science (New York, N.Y.), 292*(5514), 107-110. doi:10.1126/science.1057987

Tataranni, P. A., Larson, D. E., Snitker, S., & Ravussin, E. (1995). Thermic effect of food in humans: Methods and results from use of a respiratory chamber. *The American Journal of Clinical Nutrition, 61*(5), 1013-1019.

Telama, R., & Yang, X. (2000). Decline of physical activity from youth to young adulthood in finland. *Medicine and Science in Sports and Exercise, 32*(9), 1617-1622.

Thannickal, V. J., & Fanburg, B. L. (2000). Reactive oxygen species in cell signaling. *American Journal of Physiology.Lung Cellular and Molecular Physiology, 279*(6), L1005-28.

Tuomilehto, J., Lindstrom, J., Eriksson, J. G., Valle, T. T., Hamalainen, H., Ilanne-Parikka, P., et al. (2001). Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *The New England Journal of Medicine, 344*(18), 1343-1350. doi:10.1056/NEJM200105033441801

Tzivion, G., & Hay, N. (2011). PI3K-AKT-FoxO axis in cancer and aging. *Biochimica Et Biophysica Acta, 1813*(11), 1925. doi:10.1016/j.bbamcr.2011.08.014

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39*(1), 44-84. doi:10.1016/j.biocel.2006.07.001

van de Laar, R. J., Ferreira, I., van Mechelen, W., Prins, M. H., Twisk, J. W., & Stehouwer, C. D. (2011). Habitual physical activity and peripheral arterial compliance in young adults: The amsterdam growth and health longitudinal study. *American Journal of Hypertension, 24*(2), 200-208. doi:10.1038/ajh.2010.201

van der Vusse, G. J. (2009). Albumin as fatty acid transporter. *Drug Metabolism and Pharmacokinetics, 24*(4), 300-307.

Van Remmen, H., Salvador, C., Yang, H., Huang, T. T., Epstein, C. J., & Richardson, A. (1999). Characterization of the antioxidant status of the heterozygous manganese superoxide dismutase knockout mouse. *Archives of Biochemistry and Biophysics, 363*(1), 91-97. doi:10.1006/abbi.1998.1060

Van Remmen, H., Williams, M. D., Guo, Z., Estlack, L., Yang, H., Carlson, E. J., et al. (2001). Knockout mice heterozygous for Sod2 show alterations in cardiac mitochondrial function and apoptosis. *American Journal of Physiology.Heart and Circulatory Physiology, 281*(3), H1422-32.

Walshe, C. M., Odejayi, F., Ng, S., & Marsh, B. (2009). Urinary glutathione S-transferase as an early marker for renal dysfunction in patients admitted to intensive care with sepsis. *Critical Care and Resuscitation : Journal of the Australasian Academy of Critical Care Medicine, 11*(3), 204-209.

Wang, Z. Q., Bell-Farrow, A. D., Sonntag, W., & Cefalu, W. T. (1997). Effect of age and caloric restriction on insulin receptor binding and glucose transporter levels in aging rats. *Experimental Gerontology, 32*(6), 671-684.

Weber, J. M. (2011). Metabolic fuels: Regulating fluxes to select mix. *The Journal of Experimental Biology, 214*(Pt 2), 286-294. doi:10.1242/jeb.047050

Williams, M. D., Van Remmen, H., Conrad, C. C., Huang, T. T., Epstein, C. J., & Richardson, A. (1998). Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. *The Journal of Biological Chemistry, 273*(43), 28510-28515.

Wolden-Hanson, T., Mitton, D. R., McCants, R. L., Yellon, S. M., Wilkinson, C. W., Matsumoto, A. M., et al. (2000). Daily melatonin administration to middle-aged male rats suppresses body weight, intraabdominal adiposity, and plasma leptin and insulin independent of food intake and total body fat. *Endocrinology, 141*(2), 487-497.

Yin, F. C., Spurgeon, H. A., Rakusan, K., Weisfeldt, M. L., & Lakatta, E. G. (1982). Use of tibial length to quantify cardiac hypertrophy: Application in the aging rat. *The American Journal of Physiology, 243*(6), H941-7.

Yuen, J. W., & Benzie, I. F. (2003). Hydrogen peroxide in urine as a potential biomarker of whole body oxidative stress. *Free Radical Research, 37*(11), 1209-1213.

Zhang, F., Lau, S. S., & Monks, T. J. (2011). The cytoprotective effect of N-acetyl-L-cysteine against ROS-induced cytotoxicity is independent of its ability to enhance glutathione synthesis. *Toxicological Sciences : An Official Journal of the Society of Toxicology, 120*(1), 87-97. doi:10.1093/toxsci/kfq364

Zhang, Y., Ikeno, Y., Qi, W., Chaudhuri, A., Li, Y., Bokov, A., et al. (2009). Mice deficient in both mn superoxide dismutase and glutathione peroxidase-1 have

increased oxidative damage and a greater incidence of pathology but no reduction in longevity. *The Journals of Gerontology.Series A, Biological Sciences and Medical Sciences, 64*(12), 1212-1220. doi:10.1093/gerona/glp132

Zoncu, R., Efeyan, A., & Sabatini, D. M. (2011). mTOR: From growth signal integration to cancer, diabetes and ageing. *Nature Reviews.Molecular Cell Biology, 12*(1), 21-35. doi:10.1038/nrm3025