COMPLEX DIETARY INTERVENTIONS TO SLOW RATES OF AGING
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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements of the Degree Doctor of Philosophy

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ABSTRACT

Aging erodes motivation, cognition, sensory modalities and physical capacities, effectively depleting quality of life. Declining sensory, cognitive and motor function are reliable biomarkers of aging and mortality risk. These declines are associated with widespread dysregulation of systemic and cellular processes. We developed a complex dietary supplement (DSP) designed to ameliorate five mechanisms implicated in aging (oxidative stress, inflammatory processes, mitochondrial function, insulin resistance and membrane integrity). Remarkably, normal mice fed the DSP retained youthful functionality into old ages, reflecting slower aging rates. Marked improvements in motor function, memory capacity, spatial learning, muscle strength, visual acuity, olfaction, fecundity and important behavioral functions were observed in aging supplemented mice. Conversely, untreated control animals showed strong age-related declines in all of the above. Functional improvements were closely associated with reduced oxidative damage, elevated mitochondrial activity, positive cellular energy balance, improved glucose tolerance, boosted neurotransmitters, greater synaptic density and higher neuronal numbers in key regions of the brain. The latter was attributed to enhanced neuroprotection as well as neurogenesis. A 30% reduction in cancer rates was also documented for DSP treated p53+/- mice. The vast functional benefits greatly exceed the modest longevity extension (11%) in normal supplemented mice. For aging humans, maintaining functionality and performance into later years may provide greater social, economic and health benefits than simply prolonging lifespan. Implications of these findings extend to common age-related pathologies including dementia and neurodegenerative disease (Parkinson’s, Alzheimer’s, Huntington’s, ALS), diabetes, cancer, sarcopenia and age-related macular degeneration. Although identifying the role of specific ingredients and interactions remains outstanding, results provide proof of principle that complex dietary cocktails can powerfully ameliorate biomarkers of aging and modulate mechanisms considered ultimate goals for aging interventions.
ACKNOWLEDGMENTS

First and foremost I would like to extend a special thanks to my Ph. D. committee members: Dr. Grant McClelland, Dr. Henry Szechtm and especially my supervisor Dr. C. David Rollo. David possesses a truly brilliant scientific mind and has been a wonderful mentor to me and to all of his students. I happily admit that all academic discipline, critical scientific reasoning, and scholarly writing skills I hold today owe entirely to David’s instructional proficiency. I look forward to the opportunity of engaging in further research ventures with David in the future.

A special recognition is extended to all collaborators whose contribution to this work was absolutely imperative. The names and affiliations of collaborators appear in the author list in the appropriate chapters. When reading the list of authors I urge you to recall my sincere appreciation of their contribution to this work.

I express my gratitude to all students, volunteers and technicians who greatly assisted with experiments, lab work and animal care. Their names and contributions appear in the ‘acknowledgments’ sections following each chapter. Please take the short time required to read these brief sections.

I would like to convey my appreciation to the entire administrative team of our Biology Department. I thank Barb Reuter for always being on the lookout for my best academic interests and Karen Haines for being most helpful with all clerical needs I managed to generate.

I wish to express much thanks to Amber Faraday for looking out after the well-being of our laboratory mice. Working with Amber was an enjoyable experience.

I gratefully share the credit for my research achievements with the original inventors of our complex anti-aging dietary supplement: Jennifer Lemon, Douglas Boreham and David Rollo.

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DECLARATION OF ACADEMIC ACHIEVEMENT

INTRODUCTION and CONCLUSION: Unless otherwise referenced all ideas expressed are of Vadim Aksenov\(^1\). Original formulation of the complex dietary supplement was invented by C David Rollo\(^1\), Jennifer A Lemon\(^2\) and Douglas R Boreham\(^2\) (2000–04). Subsequent reformulations of the complex dietary supplement were put together by C David Rollo and Vadim Aksenov (2006–12).

CHAPTER 1: [Dietary Amelioration of Locomotor, Neurotransmitter and Mitochondrial Aging]

Breeding and husbandry of mice were performed by Vadim Aksenov under supervision of C David Rollo (2005–08). Preparation and administration of dietary supplement was done by Vadim Aksenov (2005–08). Behavioral time budget study was conducted by Vadim Aksenov (2006–07). Analysis of neuropeptide Y mRNA in cortex and striatum was done by Sonali Lokuge\(^1\) and Jane A Foster\(^3\) (2008) from brain samples collected by Vadim Aksenov. Quantification of neuropeptide Y mRNA density was performed by Vadim Aksenov (2008) from digital slides provided by Sonali Lokuge and Jane A Foster. Assessment of mitochondrial complex III activity and slot-blot assays of protein carbonyls in brain homogenates and brain mitochondria were carried out by Jiangang Long\(^4\) and Jiankang Liu\(^4\) (2008) from brains harvested by Vadim Aksenov. Experimental design, data analyses and statistical testing were done by Vadim Aksenov assisted by C David Rollo (2005–09).

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CHAPTER 2: [A Complex Dietary Supplement Augments Spatial Learning, Brian Mass, and Mitochondrial Electron Transport Chain Activity in Aging Mice]

Breeding and husbandry of mice were performed by Vadim Aksenov under supervision of C David Rollo (2005–10). Preparation and administration of dietary supplement was done by Vadim Aksenov and Sarthak Matravadia¹ (2006–10). Experimental approach was designed by Vadim Aksenov. The Morris Water Maze experiment was carried out by Vadim Aksenov and Parul Khanna⁵ and analyzed using Noldus© EthoVision image tracking software made available by Henry Szechtman⁶ (2009–10). Quantification of mitochondrial complex IV activity was performed by Jiangang Long and Jiankang Liu (2008) from brain samples collected by Vadim Aksenov. Measurement of brain and body mass and all statistical analyses were done by Vadim Aksenov (2009–10). Data presentation was finalized by Vadim Aksenov and C David Rollo (2010).

CHAPTER 3: [A Complex Dietary Supplement Augments Sensory and Behavioral Function, Modulates Anxiety and Prevents Neuronal Atrophy in Aging Mice]

Breeding and husbandry were performed by Vadim Aksenov supervised by C David Rollo (2006–11). Preparation and administration of dietary supplement was done by Vadim Aksenov (2006–11). Behavioral experiments including: visual acuity, step-down, open field, rotarod and circle-run⁷ were done by Vadim Aksenov (2007–11). Open field and circle run data were analyzed on Noldus© EthoVision software provided by Henry Szechtman (2008). Somatosensory tests were conducted by Vadim Aksenov (2006–08). Tests for olfactory acuity were designed by C David Rollo and Vadim Aksenov and carried out by Renata Samigullina⁵ and Vadim Aksenov (2011–12). Histological analysis of retina (staining and slide preparation) was conducted by Sergei Aksenov⁸ (2011) on eyes harvested by Vadim Aksenov. Cerebellum and olfactory bulb histology

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³ A novel behavioral test: Idea, design and apparatus construction by Vadim Aksenov
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was performed by Sergei Aksenov and Vadim Aksenov supported by William H Rodgers (2012–13) from gross samples prepared by Vadim Aksenov. All illustrations and statistical analyses were completed by Vadim Aksenov (2007–13).

CHAPTER 4: [Age-Related Declines in Brain ATP and Motor Function are Ameliorated by a Complex Dietary Supplement: Mitochondria, Free Radical Processes and Thermogenesis]

Breeding and husbandry were performed by Vadim Aksenov supervised by C David Rollo (2005–10). Preparation and administration of dietary supplement was done by Vadim Aksenov (2005–10). Behavioral tests of locomotor intensity (running distance and running speed) were conducted by Vadim Aksenov (2006–07). Grip strength and body temperature measures were collected by Vadim Aksenov (2006–09). Hydrogen peroxide in brain was quantified by Vadim Aksenov (2010–13). Levels of ATP in brain homogenates were assayed by Vadim Aksenov (2012–13). Quantification of mitochondrial complex I and II activity was done by Jiangang Long and Jiankang Liu (2008) from brain samples collected by Vadim Aksenov. Statistical analyses were prepared by Vadim Aksenov (2009–13).

CHAPTER 5: [Reduced Blood Glucose, Enhanced Exercise and Shorter Sleep in Normal and Accelerated-Aging Mice: Effects of a Complex Dietary Supplement]

Breeding and husbandry were performed by Vadim Aksenov supervised by C David Rollo (2005–10). Preparation and administration of the complex dietary supplement was done by Vadim Aksenov (2005–10). All behavioral time budget tests were carried out by Vadim Aksenov (2006–08). Food consumption was measured by Vadim Aksenov (2009). Blood glucose measurements were done by Vadim Aksenov assisted by Aleksey Matviyenkov (2009–2010). Sleep duration was recorded by Vadim Aksenov (2006–2010). Statistical tests and data presentation were assembled by Vadim Aksenov (2011).
CHAPTER 6: [A Complex Dietary Supplement Enhances Object Recognition Memory in Middle-Aged Mice in Association with Higher Neuronal and Synaptic Density]

Breeding and husbandry of mice were performed by Vadim Aksenov under supervision of C David Rollo (2009–12). Preparation and administration of dietary supplement was done by Vadim Aksenov (2009–12). The novel object recognition (memory) test was conducted by Vadim Aksenov (2010–11) assisted by Jiawei Han10. Levels of PSD-95 in brain cortex were quantified by Chiara Nicolini11 assisted by Vadim Aksenov from brains provided by Vadim Aksenov (2012–13). Preparation of histological slides (hippocampus and striatum), staining and digital imaging was performed by Sergei Aksenov and Vadim Aksenov supported by William H Rodgers (2012–13). Analyses of neuronal density and cell counts were done by Vadim Aksenov (2013) from images provided by Sergei Aksenov. Experimental design and statistical tests were completed by Vadim Aksenov (2010–2013).

CHAPTER 7: [Effects of Complex Dietary Supplementation on Tumorigenesis in p53+/- Mice]

Husbandry of p53+/- mice was carried out by Vadim Aksenov (2010–11). Male p53+/- mice were donated by Douglas R Boreham (2010). Preparation and administration of dietary supplement was done by Zoya Tov10 and Vadim Aksenov (2010–12). Survival and longitudinal body mass data were collected by Vadim Aksenov and Zoya Tov (2010–12). Harvest of tissues and organs and record of gross pathological observations were performed by Vadim Aksenov (2011–12). Screening and shipment of samples for histopathological examination were facilitated by Mary Ellen Cybulski12 (2012). Daily dietary consumption for each supplemented mouse was monitored and recorded by Vadim Aksenov (2010–12). Analysis and presentation were done by Vadim Aksenov with contribution from C David Rollo (2012).

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CHAPTER 8: [The Impact of Life-Long Treatment with a Complex Dietary Supplement on Growth, Reproduction and Aging of Mice]

Breeding and husbandry were performed by Vadim Aksenov supervised by C David Rollo (2006–11). Preparation and administration of dietary supplement was done by Vadim Aksenov and Zoya Tov (2006–11). Growth rates and anatomical features were recorded and analyzed by Vadim Aksenov (2009–11). Sleep duration, paper shredding, female fecundity and litter sizes were assessed by Vadim Aksenov (2006–11). Statistical evaluations and data interpretation were done by Vadim Aksenov (2010–12). C David Rollo contributed to theory.
PREFACE

Dear Reader,

This manuscript is prepared in a “sandwich” format. Chapters are written in journal article style and each chapter is a stand-alone piece assuming no prior knowledge of other chapters. A brief introductory statement preceding each chapter provides a transition.

To simplify the reading process, all figures, references and appendices pertaining to a chapter are included at the end of that chapter; as opposed to pooling visual and auxiliary information for the entire manuscript into a separate section. This should facilitate quicker access to relevant information without interrupting the reading flow.

Chapters 1 and 2 are replicated verbatim from published articles. Copyright information is given on the first page of these chapters. Citations and pagination were formatted to fit the overall presentation style of this manuscript.

Throughout the thesis, chapters are cross-referenced directly by chapter number (e.g., [Ch. 3] or [see: Ch. 4–6]) with the exception of published chapters 1 and 2. These chapters are cited using a number format (e.g. [16]) corresponding to a list of references (i.e. like all other cited works). The numerical citations corresponding to chapters 1 and 2 may differ between chapters based on the order in which these citations appear in text.

Every chapter opens with a detailed introduction of background specific to the following results and discussion. Therefore, to minimize repetition, the main introductory section is limited only to general background.
INTRODUCTION

This work examines the effects of complex dietary supplementation on a wide range of behavioral, physiological, anatomical and molecular biomarkers of mammalian aging. Longevity extension remains an important focus; however, the main objectives converge on preservation of youthful functionality into old age.

Experimental findings in mice are presented here with emphasis on translation to humans. Particular focus extends to brain aging and the associated sensory, motor and cognitive decline. Impacts of supplementation on tissue and cellular targets are supported by extensive behavioral assessment. Results presented throughout this work have strong implications for human aging and common age-related conditions such as: neurodegenerative disease (Parkinson’s, Alzheimer’s, Huntington’s, ALS), dementia, bradykinesia (declining locomotor activity), type 2 diabetes, cancer, cardiovascular pathology, sarcopenia (muscle loss) and loss of vision, olfaction and motor coordination.

Detailed introductory sections in each chapter provide the important background specific to experiments and findings presented in this work. This general introduction briefly outlines the current understanding of aging and the associated mechanisms and phenotypes. Our complex anti-aging dietary supplement is presented with a description of biological targets and rationale of the design. Aspects pertaining to human applications are considered. Finally, a brief description of our animal models is presented.

* * *

Understanding the aging process: Extensive comprehension of the processes accompanying aging has greatly advanced our understanding; however, the ultimate causes of aging remain unknown. Aging is understood as an extremely complex and
heterogeneous process. The mechanisms implicated in aging are tightly inter-related, casting credible doubt on the prospect of isolating a particular definitive cause. The search for a single cause (e.g. a gene or a regulatory signal) has been replaced with more comprehensive approaches.

A wide range of explanations for aging has been proposed encompassing evolutionary and mechanistic theories. Virtually every important biological discovery inspired a new cluster of theories of aging [1]. Some of the better recognized examples include the free radical (and mitochondrial) theory [2,3], the disposable soma theory [4–6], cell senescence theory (limited replicative capacity) [7,8] and the gene regulation theory of aging [9]. These and other theories are supported to various degrees by observational and/or experimental evidence (for detailed reviews see: [1,10,11]). However, a better grasp of the entire paradigm of aging emerges from exploring a combination of theories, many of which complement each other by emphasizing parallel mechanisms [1].

For example, early gene regulation theories of aging proposed that growth, maturity and senescence proceed in this order due to sequential activation and suppression of specific genes [9]. In recent decades, the genetics of longevity took on a much broader form becoming a major branch of aging research [12–14]. Some genes implicated in aging (including the p53 tumor suppressor gene [see: Ch. 7]) induce cell-cycle arrest [15] promoting replicative senescence [16]. This is highlighted by the cell senescence theories [7,8,10]. Growth and stress resistance pathways involve gene transcription regulators impacting aging rates by interfacing with nutrient, energy and stress signals [see: Ch. 8]. Accumulation of damage from oxidative respiration (mitochondria) and other endogenous and exogenous sources contributes to aging as outlined by the free radical theory [2,3]. Oxidative processes modulate gene expression [examples in Ch. 5,7,8] and directly impair cellular function contributing to cell loss and tissue and organ frailty accompanying aging. The disposable soma theory argues that animals can theoretically maintain somatic repair indefinitely by maximizing resource allocation to protective and repair systems [6]. However, the required withdrawal of resources from important functions (growth, behavior, reproduction) is unfavorable, and high investment in
somatic maintenance will eventually be wasted when the animal dies from exogenous causes [6]. The optimal allocation strategy (i.e. distribution of resources between cellular processes) depends on orchestration of gene expression, which is ultimately modulated by central control systems, cellular redox (i.e. free radicals) and energy balance (see above). The immune system is energetically costly to maintain and the associated inflammatory processes additionally contribute to accumulation of oxidative stress [17]. In this fashion, aging involves a collection of integrated mechanisms spanning the entire range of an organism’s biological organization.

From an intervention standpoint, however, understanding of theoretical models may be of secondary importance to understanding the actual cellular and physiological processes reliably associated with aging. The fundamental requirement for a successful intervention pivots on identifying affectable biological targets specific to the aging process. In addition, the complexity of mechanisms contributing to aging demands a similarly complex approach to intervention.

**The complex anti-aging dietary supplement:** Based on current gerontological literature we identified five major underlying processes implicated in mammalian aging including: oxidative stress, inflammation, mitochondrial function, insulin sensitivity and membrane integrity. These targets are linked to a wider range of cellular and systemic processes that ultimately impact aging rates and functionality (detailed discussion is presented throughout this work).

Aging rates widely vary among individuals implying considerable plasticity for exploration by dietary interventions. We designed a complex dietary supplement (DSP)\textsuperscript{13} containing 30 ingredients targeting the five aforementioned age-related processes. These processes show close interactions whereby the status of any single process can affect the status of the others, and vice versa. Thus, the principle behind a multi-ingredient (multi-targeted) approach reflects the complexly integrated mechanisms attributed to aging. Unlike single or few-ingredient supplements, a complex dietary approach allows for

\textsuperscript{13} Composition and dosages of the DSP are found in Table 1 [Ch. 1]. Minor adjustments were made to the original composition as described in [Ch. 4 and 7]. Specific biological actions (age-related targets) for each ingredient are detailed in [18] and [Ch. 4 and 7].
simultaneous action on multiple targets with opportunities for synergetic and co-stimulatory effects. Consequently, where simpler formulations fall short, our complex diet emerges as a powerful anti-aging intervention.

**Human applications:** Our objectives included designing an effective and accessible intervention delivering real sizable benefits to the general human population in the current global socioeconomic context. Our DSP is composed of minerals, vitamins, micronutrients, herbal extracts and other nutraceuticals safe for human consumption\(^{14}\) and available at health food stores. Exclusion of pharmaceutical ingredients eliminates the added costs and constraints related to prescription medication. The entire supplement can be reduced to a few capsules that can be easily ingested on a daily basis. Dosages tested on mice reflect recommended daily human doses\(^ {15}\).

In addition, adjusting dietary or lifestyle habits is not required for attaining benefits of supplementation. It is well established that healthy food choices, physical exercise and social networking substantially delay onset of functional aging and positively impact lifespan [19–22]. However, many elderly persons are often unable or reluctant to initiate substantial lifestyle changes. Adopting healthy diets, committing to regular exercise or expanding social interactions requires considerable effort and dedication; whereas noticeable improvements may be slow and loss of motivation may eventually prevail. Conversely, an intervention in the form of a dietary supplement provides a simple and virtually effortless alternative less likely to be abandoned. Moreover, further benefits may be possible by combining supplementation and active living.

**Functional aging and longevity:** Caloric restriction (usually 60–70% of *ad libutum* food consumption) is the best known intervention for slowing aging in a variety of animals [23]. However, caloric restriction requires strict fasting that may be challenging for humans. We show that our DSP in many ways mimics effects of caloric restriction

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\(^{14}\) Separately, or in limited combination (e.g., multivitamins), all ingredients in our DSP are approved for human consumption. However, our complex combination has not been tested on humans. Given the strong physiological impacts of our DSP, human trials are needed to confirm safety and establish possible health-related considerations.

\(^{15}\) Concentration of active ingredients used here (see: Table 1 [Ch. 1]) were adjusted for smaller size and higher metabolic rates of mice.
without reducing food intake. The longevity reported for DSP treated mice (11–28%)\textsuperscript{16} [24] is smaller than for calorically restricted animals; however, the range of functional benefits attained by the DSP is arguably greater (comparisons are made in [Ch. 5 and 8]). In accordance with principles of resource allocation [25], functionality is traded off against longevity assurance. Thus, vast functional improvements in supplemented mice likely limited further increase of lifespan.

In addition, caloric restriction negatively impacts growth (body size) and fecundity, while our DSP does not alter body size and actually improves fecundity of aging mice [see: Ch. 8]. Exceptionally long-lived mice (Ames and Snell dwarves) have twice the lifespan of normal mice (exceeding extension by caloric restriction) but show deficits in physical activity [26] in addition to reduced fecundity and growth [see: Ch. 5]. Conversely, aging mice supplemented with our DSP show significant improvements in physical function [Ch. 1,3–5], cognitive ability [Ch. 2 and 6] and sensory systems [Ch. 3]. For humans, the quality of life associated with preserving functionality into older ages is more desirable than adding years of decrepitude.

**Principles of experimental design:** Our experimental protocols include extensive behavioral assessment in addition to documenting impacts on cellular, anatomical and physiological biomarkers. Assessment of cellular and molecular markers is of central importance to understanding the underlying mechanisms of aging as affected by various treatments. However, when considering anti-aging supplementation (such as our DSP), individuals may have little interest in impacts on molecular biomarkers unless this ultimately translates into actual noticeable functional improvement and slower aging. Throughout this work, we document the extent of motor, cognitive, sensory and reproductive function improvement in old and aging mice. In addition, we show impacts on cancer rates, muscle strength, blood glucose and neuronal numbers in brain. These findings can be readily appreciated by all persons irrespective of scientific background.

In conjunction with previously reported lifespan increase [24], our results provide proof of principle that complex dietary formulations can powerfully ameliorate a range of

\textsuperscript{16} 11\% extension in normal mice and 28\% extension in growth hormone transgenic mice [24].
reliable biomarkers of functional aging with strong implications for common human age-related conditions.

**Animal Models:** Our DSP was tested on hybrid C57BL/6×SJL background for both normal and growth hormone transgenic (Tg) mice (Fig. 1). We employ a full range of ages whenever possible. Interpretation of age-related regressions yields a more powerful analysis than a cross-sectional group comparison. Since aging is a gradual process, effects of treatment often emerge as age-related interactions. Also, comparative analysis of slopes provides valuable information on the rates of parameter changes with respect to age.

Tg mice have rat growth hormone fused to metallothionein promoters [27]. This leads to over 100-fold chronically elevated plasma growth hormone levels [28]. As a result, Tg grow to twice the size of normal mice (see: Fig. 1 on following page) and express accelerated aging and a variety of amplified progeroid aging phenotypes. Tg mice show elevated free radical processes [29], rapid cognitive deterioration (despite superior learning ability in youth) [28], reduced locomotor function [30], altered feeding regulation [31], decreased thermogenesis [32] and altered behavioral patterns [33]. Because age-related processes are exaggerated in these animals we anticipated that impacts of supplementation may be stronger and better resolved in Tg mice. Also, the shorter lifespan of Tg allows for faster study completion. However, we found that greater impacts were often observed in normal mice. Therefore, experiments were primarily conducted in normal animals. Whenever feasible, data from Tg animals were also collected to confirm finding in normal mice.

To test effects of the DSP on cancer rates we employed a p53+- mouse model. A thorough description of this mouse is provided in Chapter 7.
Figure 1. A normal sized C57BL/6×SJL mouse (right) and a giant growth hormone transgenic mouse (left) (age-matched males: ~8 months old)

REFERENCES

CHAPTER 1

Chapter introduction:

This chapter illustrates the effects of our DSP on spontaneous locomotion in aging normal and growth hormone transgenic mice. Locomotor decline is a reliable biomarker of aging across a variety of species. Untreated normal mice showed the expected age-related decline, while supplemented animals retained youthful locomotor function into oldest ages. Assessment of locomotor duration was part of a major 24 hour time budget study in 40 mice. Impacts on other behaviors observed in the time budget experiment relevant to aging are reported in subsequent chapters. This work was my first primary author publication and solidified valuable collaborative relationships.

In collaboration with Dr. Jiankang Liu and Dr. Jiangang Long from Jiaotong University (Xi’an, China) we measured activity of mitochondrial complexes I–IV and levels of protein carbonyls (markers of oxidative protein damage) in brains of aging mice. Remarkably, supplemented mice showed a strong age-related elevation in complex III activity correlated with lowest levels of protein carbonyls. The critical significance of this finding is discussed with respect to locomotor function and aging overall.

Previous studies (work by: Lemon JA, Rollo CD, Boreham DR; McMaster Univ.) found amelioration of cognitive decline in growth hormone transgenic mice and longevity extension by the DSP. In conjunction with these reports, present data provide convincing evidence that complex dietary cocktails can powerfully impact important biomarkers of aging. This publication attracted much attention from local and global media resulting in newspaper, radio and television interviews.

Previously, our DSP was tested on transgenic mice, and we intended to use these animals as the chief murine aging model for our research. In the present study, however, stronger impacts were observed in normal animals. As a result, we shifted greater focus onto normal mice, which eventually replaced the transgenics as the principle mouse strain for studying effects of our DSP. While the growth hormone transgenic mouse is an accepted model of aging, some critics express skepticism regarding translation of benefits to wild-type animals. Confirming effects of supplementation in normal mice erases speculations of this nature.
CHAPTER 1

TITLE

Dietary Amelioration of Locomotor, Neurotransmitter and Mitochondrial Aging

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1.1 INTRODUCTION

Declining locomotor activity with age (bradykinesis) is universal from nematodes to
insects to vertebrates [1–8]. In man, declining activity contributes to ‘metabolic
syndrome’, and in advanced years, frailty. Metabolic syndrome (high abdominal fat,
insulin resistance, hypertension, arteriosclerosis and elevated free radical processes)
afflicts ~50% of North Americans >60 years of age [9]. Associated risks include type II
diabetes, stroke, heart attack and cancer [8,10]. Declining motor function also correlates
with disability risk [11]. Frailty, a leading cause of mortality and institutionalization,
afflicts 37% or more for those over 85 years [12]. Deteriorating neurotransmitter systems
mediating arousal and activity (particularly in the nigrostriatum and cerebellum) is a primary cause [8,13,14]. Aging skeletal, muscular and cardiovascular systems also contribute but exercise may be offsetting [5,6,15].

We developed a dietary supplement (DSP) targeting five key mechanisms of aging (oxidative stress, inflammation, mitochondrial function, insulin resistance and membrane integrity) [16] and tested it on normal mice (Nr) and transgenic growth hormone mice (Tg) that show greatly reduced motor activity. The growth hormone axis modulates aging [17–19] and Tg express elevated free radical processes in brain [17,20] and accelerated aging [18,19,21]. The DSP abolished age-related declines in Tg cognition, extended longevity (Nr ∼11% and Tg ∼28%) and offset radiation-induced apoptosis and DNA damage in both genotypes [16,22–24].

Here we report impacts of the DSP on motor function and the first results addressing impacts on mitochondria. The most dramatic impacts were in Nr mice. Typical of aging, the oldest untreated Nr mice showed >50% reduction in locomotion compared with their youth (∼3 h/day less movement). This was paralleled by loss of mitochondrial complex III activity and elevated protein carbonylation in brain homogenates. Mitochondria, and particularly complex III activity, are highlighted in aging and free radical generation and protein carbonyls are a recognized biomarker of free radical stress. The DSP abolished declines in daily locomotion in association with increased mitochondrial complex III activity and reduced protein carbonyls in brain homogenates. The DSP reduced the ratio of mitochondrial carbonyls to complex III activity by ∼50% in both genotypes, indicating that effective ingredients crossed the blood–brain barrier and penetrated mitochondria.

We further considered that alterations in motor activity would involve the striatum, and that growth hormones might act on striatal somatostatin neurons known to co-express neuropeptide Y (NPY) and nitric oxide synthase (NOS). We had a sensitive probe for NPY mRNA and consequently applied it in the striatum and cortex. Significant increases in NPY mRNA were found in supplemented Nr mice, but growth hormone (GH) transgenesis had no impact.

The cause(s) and amelioration of aging remain controversial, particularly with regard to
DSPs [25–29]. Our results approach dietary restriction for functional biomarkers of behavior, neurochemistry, mitochondria and oxidative stress. Functional benefits greatly exceeded the 11% lifespan extension obtained for supplemented Nr mice [22]. Prolonging youthful function in populations with expanding elderly complements, however, may be of greater value than simply extending decrepit lifespans. The DSP strongly ameliorated normal functional aging and is likely to benefit age-associated pathologies. Moreover, supplements do not require drastic dieting or induction of states mimicking diapause or dwarfism.

1.2 METHODS

Animals and diets: Breeding and husbandry of random bred C57BL/6J*SJL Nr and Tg mice were previously described [16,30]. Protocols adhered to Canada Council on Animal Care guidelines. Our DSP contained 30 ingredients available without prescription (Table 1). Dosages were derived from recommended human doses adjusted for body size and the 10-fold higher metabolic rate of mice [16]. A slurry of the DSP was soaked onto small pieces of bagel. Mice from the breeding colony were randomly assigned at weaning and for life to either the DSP treatment group (one dose/day) or remained untreated. Bagel bits were avidly eaten ensuring accurate dosing. Nr and Tg mice in appropriate age ranges were randomly selected from control and supplemented populations for various assays.

Behavioral assessment: The duration of total daily movement was assessed in transparent acrylic arenas. Four inter-connected chambers contained a running wheel, a jar with nesting material, a food dish or water bottle [31] (diagram: Fig. A1 in Ch. 5 Appendix). Mice were acclimated for 48 h (12 h light/12 h dark photoperiod) then individually videotaped for 24 h with an infrared-sensitive camera [31]. Each mouse was used only once. Groups consisted of nine supplemented and nine untreated Nr male mice ~3 to 30 months old, and 11 supplemented and 11 untreated male Tg mice ~3–15 months old. Mice were screened for health, vision and auditory responsiveness. Duration moving about the arena, wheel running and climbing on the roof were counted at one second
intervals over 24 h, and then added to obtain duration of total daily movement. Each point in Figure 1 is one mouse.

**Neuropeptide Y:** DSP impacts on motor activity were likely to involve striatum which contains somatostatin neurons likely to be affected in Tg. We had a sensitive probe for NPY which is co-localized with somatostatin in striatum so we quantified NPY in striatum and cortex. Samples were obtained from eight supplemented and five untreated Nr female mice (5–25 months old) and six supplemented and eight untreated Tg females (5–15 months old). Mice were decapitated during the mid-photophase; brains were removed on ice and immediately placed in isopentane (-60°C) for 5 s and stored at -80°C.

Two coronal brain slices (10 µm thick and 100 µm apart) were obtained by cryostat at Bregma 0.98 [32] and thaw-mounted onto gelatin-coated slides. NPY ribonucleotide antisense probes labeled with 35S radioisotope were applied [33,34]. Slide-mounted sections were fixed in 4% formaldehyde in phosphate-buffered saline, acetylated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine-HCl (pH 8.0), dehydrated in increasing concentrations of ethanol and delipidated with chloroform. Tissue sections were hybridized (approximately 500,000 counts per minute (CPM)/section) for 18 h at 55°C in a humidified chamber with radiolabeled riboprobe diluted in hybridization buffer (0.6 mol/L NaCl, 10 mmol/L Tris pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid (EDTA) pH 8.0, 10% dextran sulfate, 0.01% sheared salmon sperm DNA, 0.5% total yeast RNA, type XI, 0.01% yeast transfer RNA and 1× Denhardt’s solution). Slides were washed in 20 µg/mL ribonuclease solution for 30 min to reduce non-specific binding followed by 1 h each in 2× saline sodium citrate (SSC) at 50°C, 0.2× SSC at 55°C and 0.2× SSC at 60°C. Slides were dehydrated through a graded series of ethanol and air dried for auto-radiography. Hybridization of target tissues with an S35-labeled sense probe showed no signal, confirming specificity.

Slides were exposed to film in X-ray cassettes (Kodak BioMax MR® film, Eastman Kodak, Rochester, NY, USA) and developed for 18 h. All slides were processed in the same in situ hybridization experiment with the same probe and with film in adjacent cassettes at the same time. Images were digitized using a Qicam® camera. NPY mRNA
density was represented as grayscale images (ImageJ software). For statistical analyses samples from whole brain, striatum and a 300×300 pixel sample of cortex were averaged from two consecutive slices to obtain the best estimate of NPY mRNA density.

**Mitochondrial complex III activity:** Different mice were used for mitochondrial and behavioral studies. Brains were removed on ice and stored at -80°C. Mitochondria were prepared by Paula's method [35] and complex III activity was determined as described in Sun et al [36]. Numbers per group (male mice) were as follows: untreated Tg, eight; supplemented Tg, 11; untreated Nr, 10; and supplemented Nr, 11. Briefly, complex III activity was measured in a mixture containing 250 mmol/L sucrose, 1 mmol/L EDTA, 50 mmol/L KPi, 2 mmol/L KCN, 50 µmol/L cytochrome c, and 0.1% bovine serum albumin (with pH adjusted to 6.5 to reduce auto-oxidation of reduced CoQ1). The reaction was initiated with 40 µg/mL brain mitochondria and 50 µmol/L reduced CoQ1 (final concentration) and increased absorption at 550 nm was recorded for 2 min.

**Slot-blot assays of carbonyls in brain homogenates and mitochondria:** Protein carbonyls were measured by slot-blot [37] and relative density was obtained via optical scans. Measurements were made for both brain homogenates and the mitochondrial fraction. Carbonyl assays were from the same brains used for complex III assessment.

**Statistical analyses:** Analysis of covariance (ANCOVA) (age as covariate) was applied to assess impacts of genotype and diet. A multiple slopes model was employed as appropriate. ANOVA was used where age was not significant. Although genotypes were compared where appropriate, Nr and Tg mice were largely analyzed separately because different trends between genotypes otherwise reduced resolution. Post-hoc comparisons employed SNK tests as appropriate. Age-related trends were also characterized with linear and polynomial regression. Where applicable, planned comparisons were carried out with subsets of data divided into younger (<450 days) or older (>450 days) mice. A dividing point of 450 days best captured the very oldest ages of Tg and middle aged Nr. All data are described as means ± standard error. Where age was a covariate, means were calculated for a common age across groups. Analyses were performed with Statistica® software.
1.3 RESULTS

**Total daily movement:** Nr mice were much more active than Tg (pooled Nr versus pooled Tg data: p<0.001). The DSP had dissimilar age-related effects on genotypes and reduced longevity of Tg was also apparent (Fig. 1). Activity was elevated in both genotypes but especially in older Nr mice. In Tg, however, activity was higher in youth (Fig. 1). Consequently, Nr and Tg were analyzed separately.

For Nr, bradykinesis progressed from the youngest ages of untreated mice resulting in >50% decline in activity by 24 months of age (Fig. 1; regression: p<0.006). The DSP elevated activity of Nr mice at all ages and virtually abolished bradykinesis (Fig. 1; regression: p=0.702). At 24 months, activity of supplemented Nr was ~3 h/day longer than untreated Nr. This reflected that untreated Nr activity was ~66% of supplemented Nr mice at 24 months (Fig. 1). Regressions for Nr groups differed significantly in intercepts (p<0.00001) and slopes differed marginally (untreated b=-7.55; supplemented b=-1.15; p=0.08). Variance suggests that slopes would be better resolved with a larger sample size. Since slopes were only marginally resolved, we applied both multiple- and same-slope models of ANCOVA (covariate=age). Significant differences for treatment were obtained with either approach (same-slope ANCOVA: df=15, p<0.003; separate-slope ANCOVA: df=14, p<0.014). Thus, the most conservative estimate resolved a highly significant impact of the DSP on duration of daily movement in Nr mice (p<0.014).

Untreated Tg expressed chronically low activity across all ages (comparable to 30 months old untreated Nr). At four months, supplemented Tg were ~45% more active than untreated Tg. The subsequent decline in activity for supplemented Tg (slope=-13.17, p<0.006) was much steeper than for untreated Nr (slope=-7.553) and by 13 months (senescence for Tg) supplemented and untreated Tg were equally hypoactive (Fig. 1). Regression lines for Tg groups differed significantly in intercepts (p<0.00001) and slopes (untreated slope=-1.30; supplemented slope=-13.17; p<0.05). Separate-slopes ANCOVA resolved a significant impact of the DSP (p<0.015).

Total locomotion encompassed all measures of movement including exercise. Exercise duration did decline with age, but remained higher in supplemented Nr mice across all
ages (data not shown here) [see: Ch. 5]. Reductions in bouts of intense activity were offset by increases in moderate movement such that duration of total locomotion remained constant with age (Fig. 1).

**Neuropeptide Y mRNA:** Improved motor function of supplemented mice was associated with enhanced expression of NPY mRNA throughout striatum and cortex (Fig. 2). Differences in intensity, size and number of foci between supplemented and untreated Nr were visually striking (Fig. 2) and ANCOVA (separate-slopes model) detected significant impacts of the DSP in whole brain (p<0.041), striatum (p<0.04) and cortex (p<0.016) of Nr. In all three brain regions age-related increases in NPY mRNA were statistically resolved in supplemented Nr (linear regression: whole brain: r=0.733, p<0.039; striatum: r=0.719, p<0.044; cortex: r=0.796, p<0.018; n=8) but no pattern was detected in untreated Nr (linear regression: all brain regions: p>0.25). Slopes for supplemented Nr differed significantly from corresponding controls (whole brain: p<0.038; striatum: p<0.028; cortex: p<0.009). Changes paralleled increasing mitochondrial complex III activity in aging supplemented Nr mice (see: Fig. 3).

Although sample sizes were small, comparison of the oldest three Nr mice in each treatment (all >450 days old) indicated that NPY was 23% higher in the cortex of supplemented Nr (supplemented: 64.8±6.0 relative units; untreated: 49.9±6.2 relative units, n=6, p<0.04). A similar trend for Tg was not statistically resolved. NPY mRNA did not differ significantly between Nr and Tg.

**Brain mitochondrial complex III activity:** Nr and Tg showed similar changes in complex III activity with age and supplementation (Figs. 3A and B). Patterns of complex III activity with age had diametrically opposite slopes between supplemented and untreated mice. A general linear model (separate slopes) detected a powerful effect of the DSP (p<0.001) and an age*DSP*genotype interaction (p<0.004). Reciprocal slopes for complex III activity in supplemented versus untreated Nr mice were each statistically resolved and they significantly differed (untreated: slope=-0.015; supplemented: slope=0.015, p<0.00034). Such complexity meant that, depending on age, the supplement had negative (youth), neutral (one-year-old) or positive (older mice) impacts on complex
III activity relative to controls. Moreover, overall means did not reflect the remarkable degree of impact.

We expected that complex III would reflect ATP availability likely to contribute to levels of striatal and motor activity. Complex III activity in untreated Nr mice declined over 24 months to 46% of young mice, closely parallel to bradykinesis (compare Figs. 1 and 3A). Supplemented Nr mice showed a linear 56% gain in complex III activity from 2 to 24 months of age. At ~24 months supplemented Nr mice had 85% more complex III activity than untreated Nr. Although mitochondrial function was elevated in old supplemented Nr compared with controls, complex III activity was relatively reduced in young supplemented Nr (Fig. 3A). Thus, despite close association of complex III activity with locomotor declines in untreated Nr, supplemented Nr mice maintained constantly high motor activity that did not reflect rising activity of complex III (Fig. 1). Thus, there was no simple relationship between complex III activity and behavior.

Trends in Tg complex III activity were very similar to Nr mice (Fig. 3B). Regression slopes were only marginally resolved (untreated slope=-0.020; supplemented: slope=0.012, p=0.057), but suggests that impacts of the DSP were similar in both genotypes. Overall complex III activity, however, was lower than in Nr mice (reflecting an age*DSP*genotype interaction, p<0.029). Comparison of mean complex III activity indicated that overall activity in Tg was ~20% lower than in Nr mice (Tg: 16.42±0.96 versus Nr: 20.29±0.90 ng cytochrome c/min/mg protein). Although low complex III activity is consistent with low Tg locomotion, elevated motor activity in young supplemented Tg (Fig. 1) was associated with relatively lower complex III activity, similar to the pattern seen in supplemented Nr.

**Protein carbonyls in brain homogenates:** Supplemented Nr mice had 28% less protein carbonyls in brain homogenates than untreated Nr (p<0.048; Table 2), but carbonyls showed no age-related trends in either Nr groups. The levels of carbonyls in untreated Nr mice were ~30% lower than those of untreated Tg, but this did not achieve significance (Table 2). Supplemented Tg also showed a non-significant trend for reduced carbonylation in brain homogenates compared with untreated Tg (~15%; Table 2).
Combining all Tg revealed a ‘U’-shaped pattern of homogenate carbonyls with elevations in youth (under 150 days old) and (especially) in mice older than 400 days (Fig. 4). Tg carbonyls exponentially increased beyond an age of 400–450 days (Fig. 4) corresponding to the period of rapid die-off. In light of these complex aging patterns, we selected a subset of data restricted to Nr and Tg mice older than 450 days. This statistically resolved the exceptional increases in homogenate carbonyls in senescing Tg (3947.6±743.5 relative units) compared with stable carbonyl levels in aging Nr mice that were nearly 50% lower (2070.0±128.5; t-test: p<0.006).

Mitochondrial protein carbonyls: Although age-related trends were apparent in mitochondrial protein carbonyls, no groups were statistically resolved. For Nr mice, however, pooling treatments resolved a significant linear regression of mitochondrial carbonyls with age (r=0.5595; n=17, p<0.02). Age-related changes in carbonyls were further confirmed by separating pooled Nr data into young (<450 days old) and older (>450 days old) categories. Young Nr had mitochondrial carbonyls (2085±403) of only 34% of those observed in older Nr mice (6095±1003; p<0.0015). Increased mitochondrial carbonyls in aged Nr mice resembled the rapid late-life rise in Tg homogenate carbonyls, although not as severe. In contrast, untreated Tg expressed a trend for age-related decline in mitochondrial carbonyls. Analysis of variance (ANOVA) did not resolve an impact of genotype on mitochondrial protein carbonyls, but the DSP had a very powerful effect (p<0.0088). This was especially apparent in Tg where supplemented mice had carbonyl levels only ~47% of those of untreated Tg (p<0.0073) (Table 2). Nr supplemented mice had carbonyl levels only 64% of untreated Nr but this was not resolved.

Comparing protein carbonyls across the lifetime of genotypes (see: Table 2) obscures the enormity of early oxidative stress in untreated Tg. Thus, analysis of lifetime data did not differentiate genotype, but when the analysis was limited to mice younger than 450 days, highly significant impacts of genotype (p<0.0039) and a genotype*diet interaction (ANOVA: p<0.0014) emerged. SNK resolved a 2.4-fold elevation of mitochondrial protein carbonyls in untreated Tg (6026±591 relative units) compared with supplemented Tg (2501±639) or either group of Nr (i.e. all three latter groups were at least 60% lower.
than untreated Tg).

The relationship of mitochondrial activity to oxidative stress is critically related to aging. Consequently, we examined mitochondrial protein carbonyls in relation to complex III activity via regression analyses. Untreated Tg expressed strong increases in mitochondrial carbonyls with increasing complex III activity, whereas insignificant trends in supplemented Tg and either Nr group were neutral or declining. A first-order polynomial found that ~80% of the variance ($r^2=0.828$, $p<0.012$) in untreated Tg carbonyls was explained by complex III activity [38]. The strength of this result suggests that this mechanism explains accelerated aging of Tg (Fig. 5).

To further explore oxidative stress relative to complex III activity, we divided mitochondrial carbonyls by complex III activity to obtain a composite variable. An arcsine (Sqrt) transformation corrected for ratio-scale data [38]. ANOVA did not resolve genotype but diet proved significant ($p<0.028$). The relative proportion of carbonyls associated with mitochondrial complex III activity was reduced by 52% and 54% in supplemented Nr and Tg mice, respectively – virtually identical. For untreated Tg, reduction of carbonyls per unit of mitochondrial activity by the DSP was greater at higher levels of complex III activity. At highest complex III activity carbonyls in supplemented Tg were only 20% of untreated Tg (Fig. 5).

1.4 DISCUSSION

**Total locomotion:** Bradykinesis is evident in 1-year-old mice and 20-year-old humans [39,40], but supplemented Nr mice showed no decline even at 24 months (Fig. 1). Declines in untreated Nr began in youth and progressed to >50% loss of activity by 24 months (Fig. 1; regression: $p<0.006$). We know of no other treatment that ameliorates bradykinesis to this degree. Improved motor function in aging may be obtained by dietary restriction [2,7,41] or exercise (especially if coupled with N-acetyl cysteine and creatine) [15,42]. Flavonoids, antioxidants [14,43–45], L-deprenyl (monoamine oxidase inhibitor) [46,47] and environmental enrichment [26] are also beneficial. L-DOPA increases locomotion in conditions of depleted dopamine (DA) [8].
Rats supplemented with \(\alpha\)-lipoic acid and acetyl-L-carnitine showed 30% declines in activity in aged rats compared with 70% loss in controls [1,48,49]. Locomotion of young rats was increased by \(~\sim\) 32% [50]. Our DSP abolished age-related declines and boosted activity of young mice (Fig. 1). The DSP ameliorated but did not prevent declines in intense exercise [Ch. 5] implicating cardio-skeletal-muscular competence. Thus, further benefit might be obtained by exercise [8,42].

Despite amelioration of bradykinesis, mitochondrial function, oxidative damage and neurotransmitter declines, the DSP only extended Nr longevity by a modest 11% [22]. Female mice selected for high activity expressed deferred senescence but accelerated late mortality [4]. Alternatively, long-lived *Drosophila* ‘Methuselah’ mutants express oxidative stress resistance but no improvement in bradykinesis [51]. This resembles Tg where the supplement increased activity only in youth (Fig. 1) despite amelioration of cognitive aging and a 28% increase in longevity [16,22]. Dietary restriction benefits *Drosophila* in early life but negatively impact late-life stress resistance [52]. Similarly, acetyl-L-carnitine improved cognition and survivorship but not age-related sensory-motor deficits in rats [53]. Increased longevity of dwarf rats via GH manipulation was also accompanied by functional impairments [54]. Thus, aging functions are somewhat dissociable from one another and longevity [7,51,52,55,56].

**Neuropeptide Y mRNA:** NPY is co-localized in striatal interneurons with somatostatin and neuronal NOS [57–59]. We applied NPY mRNA as a biomarker for these neurons since NPY regulates foraging [60] and is altered in Parkinson's disease [61]. The DSP strongly altered NPY mRNA (Fig. 2). Similar increases occur in number, size and intensity of foci of somatostatin stimulated by GH excess in the hypothalamus [62] but NPY showed no alterations in Tg here. Bradykinesis is associated with declines in DA [8] and parallel loss of striatal NPY [63]. DA neurons are susceptible to oxidative stress which the DSP reduces [8,64]. Striatal DA of Tg is \(\sim\) 40% that of Nr, which undoubtedly contributes to hypoactivity [30]. Striatal DA and NPY neurons are closely associated and NPY regulates DA synthesis and release [65,66]. The broad distribution of NPY/NOS foci (Fig. 2) could facilitate nitric oxide (NO) release mediating general
arousal and waking [57,58]. NO inhibits DA reuptake, elevating extracellular DA [59,67]. NO promotes motor activity and can contribute to hyperactivity [68,69]. Elevation of blood flow and metabolism by NO could also be important in aging [70].

**Mitochondrial complex III activity:** Declining mitochondrial function is a biomarker of aging implicated in free radical generation [29,41,48,71,72]. Complex III activity in brains of old mice falls to ~60% of youthful levels [73] and was 46% of youth in untreated Nr at 24 months. In contrast, supplemented Nr showed remarkable 56% gains in complex III activity from 2 to 24 months of age. Tg had ~20% lower complex III activity than Nr (overall), and although complex III activity rose in supplemented Tg, levels did not exceed those of old untreated Nr (compare Figs. 3A and 3B).

The DSP increased mitochondrial activity but reduced free radical processes relative to complex III activity (see below). Increased ATP would support functionality of aging mice. Oddly, the DSP lowered complex III activity in young mice (Fig. 3), suggesting greater benefits at older ages. Mitochondrial status in youth can influence later-life functions however [74], so benefits of youthful supplementation to older ages requires assessment.

**Homogenate protein carbonyls:** Protein carbonyls reflect oxidative damage associated with enzyme dysfunction, protein accumulation, cellular inclusions, extracellular deposits, neurodegeneration and aging [37]. Oxidative stress generally increases with age although most studies do not separate cytosolic and mitochondrial compartments. The DSP reduced homogenate carbonyls in Nr but age-related trends were absent (Fig. 4). We documented increasing superoxide radical and lipid peroxidation with age in Tg and Nr mice [20], suggesting that constant carbonyls in Nr homogenate likely reflects sustained proteosome function.

Although GH can ameliorate some symptoms of aging, considerable evidence suggests GH accelerates aging [17–19,21,75,76]. Insulin-like growth factor 1 (IGF-1) and insulin signaling via the PI3K pathway modulate aging and many models of life extension (e.g. dwarf mice) express reduced PI3K signaling. Growth factors signal via free radicals and chronic low radiation stimulates growth [76]. GH, insulin and IGF-1 are elevated in Tg
and IGF-1 regulates other growth factors [17,76,77]. Oxidative stress and accelerated aging of Tg [20] are consistent with the free radical theory.

Protein carbonyls in Tg homogenates expressed a ‘U’-shaped pattern with the highest levels in youth and old age. Beyond ~400 days carbonyls rose precipitously (Fig. 4), even though some mitochondrial measures were lowest at these ages. Free radicals in Tg homogenate may involve extra-mitochondrial sources like NAD(P)H oxidase or low ATP may compromise the proteosome. Accumulation of oxidized/inactivated proteins is a reliable biomarker (and possible mechanism) of aging. Carbonyls negatively correlate with cognitive and motor functions in aging rodents and protein accumulation in aged gerbil brain were cleared by an antioxidant that restored youthful functions [43,78]. Aging is linked to capacities for repair and turnover such as autophagy, lysosomal function and proteosome activity [79]. Aging mice exhibit increases in oxidized cysteine and ubiquinated proteins whereas long-lived mole rats maintain proteosome function and forestall bradykinesia for >20 years [80]. Reversibly oxidized cysteine residues and ATP regulate enzymes and signaling cascades, so ATP shortfalls and oxidative stress in old Tg (Fig. 4) would synergistically disrupt cell functions and energy balance [8,81,82].

Tg show constitutively elevated DNA damage as indicated by γH2AX foci and 8-oxodG levels as well as hypersensitivity to radiation-induced DNA damage and apoptosis [23,24]. Knockdown of MTH1, the main sanitizing substrate for 8-oxodG (and oxidized ATP) induces replicative senescence and DNA damage even if free radicals, antioxidants and mitochondrial activity are unaltered. Thus, the nucleotide pool is also a major target of oxidation [83].

**Mitochondrial protein carbonyls:** Reduction of mitochondrial carbonyls by the DSP indicates penetration of the blood-brain barrier and mitochondria (Table 2; p<0.009), key goals of aging interventions [84–86]. Catalase expressed in mitochondria extended longevity of mice by ~17% [87]. The DSP reduced carbonyls by ~36% and ~53% in Nr and Tg, respectively. This was statistically resolved for Tg (p<0.008) but not for Nr mice (Table 2). Rising carbonyls and declining complex III activity in old untreated Nr (Fig. 3A) suggest reduced ATP production potentially impacting numerous functions. In
supplemented Nr, increased metabolic rate as reflected in higher physical (Fig. 1) and mitochondrial activity (Fig. 3) likely offset the degree of free radical reductions and limited gains in lifespan to 11%.

Differences in mitochondrial carbonyls between untreated Tg and Nr groups were not resolved across lifetimes partly because increases in carbonyls (Table 2) occurred at different ages. When analysis was limited to mice <450 days old strong genotype (p<0.004), and genotype*diet interactions (ANOVA: p<0.0015) emerged. SNK found untreated Tg (6026±591 relative units) differed from other groups that all had at least 60% fewer carbonyls (2501±639 relative units or less).

Age-related trends in mitochondrial carbonyls were not significant in either Nr group, but regression analysis of pooled data did detect increases with age (n=17, r=0.559, p<0.02). Dividing mice into those younger or older than 450 days showed that carbonyls in youth were only 34% of older mice (p<0.0017). Untreated Tg expressed high levels of mitochondrial carbonyls (Table 2), but these occurred in youth (unlike Nr). Lowest levels occurred in old Tg with low complex III activity. Early elevation of GH, anabolism and oxidative stress may reduce subsequent ATP availability and accelerate aging of Tg [17,18,75,76].

Youthful elevation of carbonyls in untreated Tg was associated with highest complex III activity (Fig. 3). Overall, however, Tg complex III activity was ~20% lower than age-matched Nr. Even the highest Tg complex III activity fell in the Nr range despite being associated with high mitochondrial carbonyls. Further analyses revealed that untreated Tg mitochondria are associated with exceptional levels of protein carbonyls that dramatically rise with increasing complex III activity (Fig. 5; r=0.91). Extra-mitochondrial sources of free radicals [88] or diversion of energy away from longevity assurance mechanisms could also contribute [75].

In some circumstances high mitochondrial activity can generate low levels of free radicals, thereby increasing ATP production without accelerating aging [15,72,89–91]. Reduced oxidative stress relative to mitochondrial activity contributes to exceptional lifespans of birds or dietary restricted rodents [17,76]. ANOVA of the ratio of
mitochondrial carbonyls to complex III activity showed that the DSP reduced the ratio from 311.3±45.9 relative units in untreated to 166.2±40.8 for supplemented mice (i.e. supplemented Nr and Tg generated ~50% fewer carbonyls per unit of complex III activity; p<0.021). For the highest complex III activity in Tg, the DSP reduced carbonyls by a remarkable 80% (Fig. 5), increased longevity by 28% [22] and elevated youthful locomotion (Fig. 1). Supplemented Tg still died earlier than Nr, however, and their bradykinesis progressed rapidly (Fig. 1). Alternatively, increased complex III activity and reduced carbonylation per unit of complex III activity in supplemented Nr likely explains how both functional gains and modestly increased longevity were obtained.

**Divergence of homogenate versus mitochondrial carbonyls:** If mitochondria are the main source of free radicals then cytosolic and mitochondrial oxidative stress should be correlated [92–94]. Instead, age-related patterns of carbonyls in homogenate and mitochondria strongly differed (Table 2; Figs. 4 and 5). Homogenates included mitochondria so estimates of divergence are conservative. Homogenate carbonyls were ~63% of those in mitochondria overall (t-test: p<0.012), but there was no correlation between homogenate and mitochondrial levels (r=0.06; p>0.05). Even reduction of mitochondrial carbonyls in supplemented Tg by ~50% was not reflected in homogenate (Fig. 4; Table 2). *Caenorhabditis elegans* also shows increased carbonyls with age in the mitochondria, but not in the cytoplasm [28], whereas long-lived MCKL1 mice express elevated oxidative stress in the mitochondria but reductions in the cytosol [95].

Cytosolic sources of free radicals may also be important. Thus, angiotensin II (a model for growth factor signaling) generates waves of free radicals derived from NAD(P)H oxidase and NOS. Resulting oxidative stress can derive mitochondrial DNA damage, mitochondrial free radical generation and ultimately, apoptosis. Thus, growth factor signaling can damage mitochondria and compromise energy production [88].

Bradykinesis is an excellent aging biomarker since it is linked to metabolic rate, feeding, fat storage, brain neurotransmitters, cardiovascular and skeletal-muscular systems and mitochondria. A linkage to brain PI3K signaling is also suggested by knockout of insulin receptor substrate-2 (a PI3K element modulated by the GH axis).
This altered energy balance and extended mouse longevity by 18%. Obesity and hyperinsulinemia were offset by increased activity, amelioration of bradykinesis at 22 months, elevated glucose utilization and reduced free radical stress [96]. Accelerated aging of Tg may involve diversion of resources away from longevity assurance systems to growth [75,97,98]. This predicted that Tg would express elevated free radicals that was strongly confirmed [20]. Complex III activity was reduced in untreated Tg indicative of energy shortfalls. Energy limitation was also suggested by dietary preferences for carbohydrate [99] and a carbohydrate-biased metabolism [21]. The levels of ATP in Tg skeletal muscle were 51% that of Nr [20]. Our results suggest that accelerated aging of Tg is strongly linked to free radical generation at complex III (Fig. 5). A universal correlate of growth factor signaling via PI3K is mammalian target of rapamycin (mTOR). mTOR modulates growth, mitochondrial activity and ATP production and is associated with reduced longevity [92]. GH/IGF-1 likely regulate mitochondrial coupling and associated free radical generation via this pathway [100]. Remarkably, the DSP elevated mitochondrial activity (energy) and reduced free radical processes, thus ameliorating two key mechanisms linked to aging and its dysregulation in Tg.

1.5 AUTHOR CONTRIBUTIONS

All participated in design, interpretation and review of the manuscript. VA and J Long equally contributed to behavioral and mitochondrial work. SL and JF conducted the neurotransmitter work. CDR, VA and J Liu wrote the paper and analyzed the data. J Liu contributed to mitochondrial aspects and CDR contributed to theory, development of the supplement and behavioral studies.
1.6 ACKNOWLEDGEMENTS

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1.7 FIGURES and TABLES

Table 1. Ingredients included in the complex dietary supplement

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Mouse dose (mg/day/100 mice)</th>
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<th>Mouse dose (mg/day/100 mice)</th>
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<td>Vitamin B₁</td>
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<td>Ginko biloba</td>
<td>18.29</td>
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<tr>
<td>Vitamin B₃ (niacin)</td>
<td>30.49</td>
<td>Ginseng</td>
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<tr>
<td>Vitamin B₆</td>
<td>60.98</td>
<td>Green tea extract</td>
<td>487.8</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.18</td>
<td>L-Glutathione</td>
<td>30.49</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>350.61</td>
<td>Magnesium</td>
<td>45.73</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.02</td>
<td>Manganese</td>
<td>19.05</td>
</tr>
<tr>
<td>Acetyl L-carnitine</td>
<td>146.45</td>
<td>Melatonin</td>
<td>0.73</td>
</tr>
<tr>
<td>Alpha-lipoic acid</td>
<td>182.93</td>
<td>N-acetyl cysteine</td>
<td>304.88</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>132.11</td>
<td>Potassium</td>
<td>18.11</td>
</tr>
<tr>
<td>Beta carotene</td>
<td>21.95</td>
<td>Rutin</td>
<td>304.88</td>
</tr>
<tr>
<td>Bioflavonoids</td>
<td>792.68</td>
<td>Selenium</td>
<td>0.05</td>
</tr>
<tr>
<td>Chromium picolinate</td>
<td>0.30</td>
<td>Vitamin E</td>
<td>326.83</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.61</td>
<td>Cod liver oil (Omega 3)</td>
<td>1219.51</td>
</tr>
<tr>
<td>Garlic</td>
<td>3.81</td>
<td>Coenzyme Q10</td>
<td>60.98</td>
</tr>
<tr>
<td>Ginger root extract</td>
<td>600.37</td>
<td>Flax seed oil</td>
<td>1219.51</td>
</tr>
</tbody>
</table>

Some dosages differ slightly form the formulation applied in earlier publications and dehydroepiandrosterone was removed [see: 16,22–24]
Table 2. Protein carbonylation of brain homogenates and mitochondria of Nr and Tg mice fed a complex dietary supplement versus untreated controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Brain homogenates (mean±SE [N])</th>
<th>Brain mitochondria (mean±SE [N])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Untreated</td>
<td>2320.4 ± 299.9, (10)*</td>
<td>4895.5 ± 1261.4, (8)</td>
</tr>
<tr>
<td>Normal</td>
<td>Supp</td>
<td>1814.9 ± 352.3, (11)*</td>
<td>3152.7 ± 770.9, (9)</td>
</tr>
<tr>
<td>Transgenic</td>
<td>Untreated</td>
<td>3415.5 ± 289.4, (8)</td>
<td>5354.6 ± 826.6, (9)†</td>
</tr>
<tr>
<td>Transgenic</td>
<td>Supp</td>
<td>2931.5 ± 296.2, (11)</td>
<td>2501.9 ± 483.9, (10)†</td>
</tr>
</tbody>
</table>

Protein carbonyls were estimated by slot-blot [see: 37] and quantified (relative density) via optical scans.

*significantly differ: p<0.048
†significantly differ: p<0.007
Figure 1. Relationship between age (months) and total movement (min/24 h) for supplemented (n=9) and untreated (n=9) Nr and supplemented (n=11) and untreated (n=11) Tg mice (p-values indicate significance of illustrated regression lines):
Nr untreated: y=432.631−7.553*x, p<0.006
Nr supplemented: y=476.808−1.150*x, p=0.702
(intercepts significantly differ: p<0.0001; slopes marginally differ: p=0.08)
Tg untreated: y=230.892−1.298*x, p=0.7758
Tg supplemented: y=385.093−13.171*x, p<0.006
(intercepts significantly differ: p<0.0001; slopes significantly differ: p<0.05)
Analysis of covariance (covariate=age) detected significant differences between supplemented and untreated Nr (p<0.014) and between supplemented and untreated Tg (p<0.02)
Figure 2. Examples of NPY mRNA expression in whole-brain cross-sections of untreated and supplemented Nr female mice at ~1.5 and ~2 years of age. Note the diffuse pattern of foci throughout the striatum and cortex and increases in their number and intensity in supplemented mice.
Figure 3. (A) Relationship between age and mitochondrial complex III activity in brains of untreated (n=10) and supplemented (n=11) Nr mice. Both regressions were significant, and slopes significantly differed, p<0.0003. Untreated: r=-0.656, linear regression: p<0.04, y=24.735–0.014*x; Supplemented: r=0.891, linear regression: p<0.0003, y=15.443+0.015*x. (B) Although similar in pattern to normal mice, linear regression models for mitochondrial complex III in supplemented and untreated 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.
Figure 4. Tg mice expressed a complex ‘U’-shaped pattern of protein carbonyls in brain homogenate with age. Dietary treatment was not resolved for Tg groups so data were pooled and analyzed as a first-order polynomial. A sharp rise in carbonyls was apparent in the oldest Tg mice. In Nr mice homogenate protein carbonyls showed no significant age-related pattern and a very weak trend for increase with age (pooled for clarity of comparison to Tg mice). The first-order polynomial fit for Tg mice was as follows: \( y=4907.9-20.09x+0.038x^2 \), \( n=18 \), \( r=0.689 \), \( p<0.009 \), where carbonyls are relative density slot-blot units, and age is in days.
Figure 5. Relationship of mitochondrial carbonyls to complex III activity in supplemented and untreated Tg mice. Regression analyses of supplemented mice did not obtain significance but the parabolic fit for untreated Tg mice was highly significant: 
\[ y = 4710.1 - 508.6 + 27.2x^2 \], \( n = 8 \), \( r = 0.9109 \), \( p < 0.012 \)
1.8 REFERENCES


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CHAPTER 2

Chapter introduction:

The previous chapter described impacts of DSP on locomotor function. Here, we document effects of supplementation on cognitive ability of aging mice. Cognitive decline reliably accompanies aging and in humans is often associated with debilitating neurodegenerative conditions. Normal mice were tested on a spatial memory task (Morris water maze). Supplemented animals expressed superior (youthful) learning at old ages (>1 year), while age-matched untreated mice were unable to learn the maze. Later [see: Ch. 6] we show that benefits of the DSP also extend to non-spatial memory (object recognition). Amelioration of cognitive decline is one of the ultimate goals for anti-aging interventions.

We also show impacts of the DSP on activity of mitochondrial complex IV. Because complex IV deficiency is closely linked to Alzheimer’s disease, results are presented here in conjunction with cognitive data (and not in the previous chapter).

Finally, we report greater brain mass in supplemented mice. Although brain mass does not directly reflect morphology or cell number, positive correlations with cognitive function are established. Histological data was not yet available, but given the cognitive, mitochondrial and protein carbonyl data [Ch. 1], neuroprotective properties of our DSP were proposed. Indeed we later report higher neuronal numbers and reduced neuronal atrophy in a number of brain regions [see: Ch. 3 and 6].

This work was published in the journal of the American Aging Association and similar to the previous publication attracted much media attention. I was later awarded a travel stipend by the American Aging Association to present these findings at an international symposium on aging in Brighton, UK.

Note: Citation number [30] refers to Chapter 1
CHAPTER 2

TITLE

A Complex Dietary Supplement Augments Spatial Learning, Brain Mass, and Mitochondrial Electron Transport Chain Activity in Aging Mice

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2.1 INTRODUCTION

Cognitive decline afflicts virtually all aging animals and in humans is also associated with neuropathologies such as Alzheimer’s (AD) and Parkinson’s (PD) diseases [1–3]. Modern industrialized societies face rapidly aging cohorts at high risk for cognitive deterioration and pathology. In the USA, AD afflicts 5.3 million (Alzheimer’s Association, 2010) and PD ~1 million people (Parkinson’s Disease Foundation). Thirteen percent of those older than 65 years of age have AD, and this is the fifth leading cause of death for this cohort (Alzheimer’s Association, 2010). By 2050, 115 million people worldwide are predicted to have AD [4]. Available treatments amount to several drugs
that provide temporary relief or slow progression [5–7]. Risk of AD may be reduced, however, by antioxidants [8–10], healthy diets [11], environmental enrichment [12,13], and exercise [14]. Preservation of cognitive function into older ages is associated with increased longevity [15]. Maintaining youthful cognitive functions into older ages would yield great benefits for quality of family life and reduce economic and social costs.

Aging humans display progressive loss of spatial navigation skills [16] accentuated in those with even mild AD [17–19]. Neurobiological and behavioral manifestations of cognitive decline relevant to humans are shared by several animal models [20] including those genetically engineered to express attributes of human pathologies such as AD [e.g., 12–14]. Aging rodents are also especially vulnerable to declining spatial memory [21], and this can be quantified by performance in the Morris water maze (MWM) [12,14,22–25]. Learning and memory processes tested by the MWM are considered to particularly assess hippocampal function [14,21,25].

Age-related accumulation of oxidative damage in brain contributes to cognitive deterioration [26–28], but recently, energy metabolism (i.e., age-related decline in mitochondrial function) has also been highlighted [29,30]. Mitochondrial complex I dysfunction is strongly implicated in PD [31], and complex III is also associated with generation of reactive oxygen species (ROS) relevant to aging [32,33]. Complex IV deficiency was recently identified in AD [34,35].

Although boosting electron transport chain (ETC) complexes could improve ATP supply, associated increases in mitochondrial respiration could also elevate oxidative stress. Ideally, effective interventions should benefit mitochondrial function while effectively ameliorating ROS damage. We developed a complex dietary supplement (DSP) designed to target five key mechanisms of aging (oxidative stress, inflammation, mitochondrial function, insulin resistance, and membrane integrity). The DSP markedly improved learning in a model of accelerated cognitive aging (transgenic growth hormone mice) [36] and extended longevity of normal mice by 11% [37]. It entirely prevented age-related declines in spontaneous motor activity and elevated brain mitochondrial function (complex III) in old age while reducing the rate of free radical generation [30]. Levels of
striatal neuropeptide Y were elevated in supplemented normal mice [30]. Additionally, the DSP protected mice from oxidative damage associated with aging and radiation [30,38,39].

Previous cognitive benefits of the DSP were demonstrated in transgenic growth hormone mice that express accelerated aging and rapid cognitive decline [36]. Here we demonstrate equally striking preservation of cognitive functions in aging normal mice using the MWM. Both supplemented and untreated young mice (<1 year old) learned the water maze well. Untreated animals more than 1 year of age, however, showed no evidence of learning even after 5 days (15 trials). In marked contrast, older supplemented mice showed ~50% better performance than age-matched untreated controls. In fact, learning performance was identical to youth, even for mice ~2 years of age. Although supplemented mice did not differ in size from controls, both genders had significantly greater mass-specific brain mass than untreated mice.

We previously reported a 56% elevation in activity of mitochondrial complex III in brains of supplemented mice by ~2 years of age [30]. Similarly, we show here that supplementation achieved a ~65% increase in complex IV activity by ~2 years of age. A correlation analysis suggested a linkage between predicted activity of either mitochondrial complexes III and IV (or their summation) and cognitive performance in aging.

These results represent “proof of principle” that complex nutraceutical formulations can profoundly ameliorate normal cognitive aging. This, combined with our earlier results with transgenic growth hormone mice (that express neuropathology similar to AD) [36], suggests promise for amelioration of human neuropathologies.

2.2 METHODS

**Animals and diets:** Breeding and husbandry of mice (random bred C57BL/6J×SJL) were previously described [36]. Protocols adhered to the Canada Council on Animal Care. Our DSP contained 30 ingredients [see: Ch. 1] available from health food stores without prescription. Daily dosages were derived from recommended human values
adjusted for body size and the higher metabolic rate of mice. Supplemented mice received 1 dose/day of DSP that was soaked onto small pieces of bagel in their home cages [details in: 30,36]. All mice avidly ate the supplement confirming no issues concerning palatability. Mice were randomly assigned at weaning and for life to either the DSP group or untreated controls. Mice (ages 2–31 months) were randomly selected from control and supplemented populations for various experiments.

**Morris water maze:** Experimental groups comprised 20 untreated and 17 supplemented mice of both genders (ages 3–25 months). Spatial learning was assessed in a circular pool (D 148cm) filled with water (27±2°C) rendered opaque by adding powdered milk. Room temperature was 23±1°C. The learning task of the mice was to locate a platform (D 11cm) submerged 1.5cm below water surface and positioned about 37cm from the pool wall. An enclosure around the pool provided distal visual cues for spatial orientation. Mice were lowered gently into the water near the wall of the pool, at two alternating starting locations: one position was in the quadrant directly across from the platform (that is, 180° to the platform), and the other position was at 90° to platform. A 120 seconds time limit was allowed to locate the platform. If unsuccessful, mice were guided to the platform and given 30 seconds to survey visual cues. Each mouse was tested over five consecutive days with three trials/day separated by a 40–50 minute inter-trial interval. A training schedule with inter-trial intervals of 10–60 min may effectively assess long-term memory acquisition in mice [40]. Between trials mice were dried and placed in cages with a heating pad. Pre-experimental swims (without platform) were conducted prior to the study to reduce emotionality associated with the novelty of swimming and to assess swimming ability (poor swimmers were excluded). Trials were videotaped from above and analyzed using Noldus© EthoVision tracking software to obtain measures of latency to reach the platform and swimming speed.

**Brain mitochondrial complex IV activity:** We were interested in complex IV activity as it is implicated in Alzheimer’s disease [34,35]. Complex III was analyzed previously [30]. Normal male mice (n=18) were used for mitochondrial studies (not tested in the MWM). Brains were removed on ice and stored at -80°C. For the analysis of complex IV
activity [41], assay buffer contained 50 mmol/l phosphate buffer, 0.1% bovine serum albumin, 0.2% Tween-20, and 60 μmol/l reduced cytochrome C. Complex IV was measured by monitoring the decrease of reduced cytochrome C at 550 nm.

**Brain weight:** Brains were collected from a wide age range (2–31 months) of normal supplemented and untreated male and female mice from the breeding colony (n=182). Mice were decapitated, brains were dissected out, and immediate wet weight was measured. Body mass of each mouse was also recorded, and brain weights were corrected for body mass (e.g., milligrams brain per gram mouse).

**Statistical analyses:** Effects of diet on learning in young and old mice in the MWM were compared using a t-test where latency is reported as mean±SE. Age-related trends in the levels of complex IV activity were described with linear regressions, and slopes and intercepts were compared using an ANCOVA with age as a covariate. Mass-specific brain weights were characterized with linear regression and impacts of diet were distinguished using t-test and ANCOVA. Analyses were performed with Statistica® software.

2.3 RESULTS

**Morris water maze:** Learning in the MWM was evaluated by measuring the latency to locate the escape platform on day 1 (trials 1–3) against day 5 (trials 13–15). Latency is reported separately for young (<1 year old) and old (>1 year old) mice to account for effects of age on learning. Mean ages for young supplemented and untreated mice were 6.2 and 6.7 months; and for old supplemented and untreated mice were 19.3 and 19.1 months of age, respectively. No gender differences were observed other than females swam on average ∼30% faster than males; however, this was not significantly resolved. Hence, data were pooled for both genders to increase statistical resolution.

As expected, all mice displayed high latency scores irrespective of age group or dietary supplementation on day 1 (i.e., no significant differences were found between young and old or between supplemented and untreated mice) (Fig. 1). Average latency for all combined groups on day 1 was 81.1±4.5s. Young mice from both treatments showed a
virtually identical decrease in latency on day 5 compared to day 1 (Fig. 1). Since diet did not affect learning in this age group, young mice were pooled to increase statistical resolution. A reduction in latency from 82.2±8.1s on day 1 to 46.7±10.1s on day 5 (improvement of 43%) was significantly resolved (p<0.02) indicating that young mice were successful at learning the maze.

In other studies, some mice employ a search strategy where they circle the pool at a distance from the wall that ensures finding the platform (chaining) [42,43]. With further training, such mice ultimately adopt a direct approach to the platform, indicating spatial learning [43]. Examination of paths for our mice suggests specific spatial learning rather than chaining (see: Fig. A1 in appendix).

Contrary to young animals, the DSP had a significant impact on learning of old mice. For old untreated mice, the latency to locate the escape platform remained largely unchanged from day 1 to day 5 (a difference of 8% was not significantly resolved) indicating that the task was not learned (Fig. 1). However, supplemented mice showed a 46% significant decrease in latency from day 1 to day 5 (75.7±8.5s to 41.3±12.5s; p<0.05) (Fig. 1). This indicates that old supplemented mice successfully learned the maze, unlike the age-matched untreated counterparts. Additionally, latency to locate the platform for old supplemented mice on day 5 (41.3±12.5s) was significantly less than of untreated old mice on the same day (77.0±10.0s; p<0.05) reinforcing the finding that learning only occurred in the supplemented group (Fig. 1). Strikingly, day 5 performance of old supplemented mice was virtually identical to that of young animals (41.3±12.5s and 46.7±10.1s, respectively) (Fig. 1). Hence, it appears that DSP supplementation preserved cognitive function of old mice at youthful levels.

We observed that some animals showed evidence of improvement, but others showed no learning at all (an all-or-nothing pattern). We tested whether the proportion of mice showing no evidence of learning differed between dietary treatments. Mean latency to locate the platform on day 1 (pre-learning trials) was 81 s for the entire population. Therefore, mice with day 5 latency scores greater than or equal to 81 s were considered non-learners since no improvement was seen; all other mice were considered to have
learnt the maze to some degree. Based on this modest criterion, ten out of 20 untreated mice (50%) did not learn the maze at all compared to only two out of 17 supplemented mice (12%). Chi-square resolved a significant difference (p<0.05) between the proportion of non-learners and learners between treatments.

**Mitochondrial complex IV activity:** Impact of the DSP on brain mitochondrial complex IV activity was assessed with respect to age. Regression lines relating complex IV activity to age had virtually opposite slopes for untreated (-0.527) versus supplemented mice (+0.665), and differences in slopes and intercepts for these regressions were statistically differentiated (ANCOVA, covariate=age, slopes: p<0.02, intercepts: p<0.0001; Fig. 2). Untreated mice had the highest levels of complex IV when young (∼100 days old) but then expressed progressive age-related loss that declined to ∼50% that of youthful levels by ∼2 years of age (Fig. 2). Conversely, supplemented mice showed a steady age-related rise in complex IV activity and by 2 years of age levels were ∼65% higher than in youth (Fig. 2). Thus, the DSP not only prevented age-related decline in brain complex IV activity but it also appears to support the increasing activity with age (Fig. 2).

These findings mirror previous results obtained for complex III activity in brain [30]. For the 18 mice in the current study, the impact of the DSP on complex III activity was more strongly resolved (ANCOVA; p<0.003) than complex IV (ANCOVA; p<0.02), but both the magnitude and direction of impacts were very similar. Given the linkage of these complexes and their association with free radical generation, we also considered the impact of the DSP on the summed activities of complexes III and IV. ANCOVA obtained a stronger statistical resolution for the complex III+IV summation than for either individual complex (p<0.0015).

**Brain mitochondrial activity and learning:** On advice during review, we explored a possible relationship between activity of brain mitochondrial complexes III and IV and performance in the water maze (impacts of DSP on age-related levels of brain complex III activity were previously reported [30]). Since measures of mitochondrial activity and learning performance were assessed in different animals, we utilized regression equations
to predict mitochondrial complex IV activity for mice of a given age in each treatment group (see: Fig. 2). We then examined correlations between predicted values of complex IV activity and learning performance in the MWM (i.e., latency to locate escape platform on day 5). The same procedure was repeated for complex III and for the sum of complexes III+IV. Table 1 shows the correlation between complexes III, IV, and III+IV and learning in the MWM. No correlation was found between mitochondrial complex activity and learning in younger (<1 year old) mice (Table 1). However, for old mice, significant relationships emerged between learning and levels of complexes III, IV, and III+IV activities (p<0.03, p<0.05, and p<0.04; respectively) (Table 1). These results suggest that older mice with higher levels of mitochondrial activity in the brain were better learners.

**Brain weight:** Mass-specific brain weight (i.e., brain weight corrected for body mass) was collected for 115 untreated mice and 67 supplemented mice of both genders. Mean ages for untreated and supplemented males and females were 17.5, 16.3, 16.8, and 16.8 months; respectively. Body mass of females was 13% less than body mass of males (p<0.00001). However, no difference in body mass was observed between supplemented and untreated mice of either gender, consistent with our previous results [36]. Thus, variation in mass-specific brain weights between treatments does not reflect differences in body mass. On average, mass specific brain weights of females were 18% greater than males (p<0.0001). Male mice did not show a significant reduction of brain weight with age in either treatment despite apparent trends (Fig. 3).

Female mice, however, showed strongly significant reductions in brain weight with age in both treatment groups (p<0.001) (Fig. 3). Female brain weights were greater in youth, but by ~2 year of age approached those of male mice (Fig. 3). The DSP was associated with significantly larger brains in both male and female mice. Since no significant age-related trends were established in males, a t-test was applied. Mean brain weights of supplemented males were 7% greater compared to untreated controls (p<0.02) (Fig. 3). For females, mean brain weights were 11% greater if supplemented compared to untreated controls (t-test: p<0.02) (Fig. 3). Since weight of female brains was strongly correlated
with age, ANCOVA (covariate=age) was also applied confirming that treatment with DSP significantly increased brain weight across all ages (p<0.001).

2.4 DISCUSSION

Morris water maze: The MWM is a standard test of memory and learning for mice and rats and is routinely applied to assess aging and pathology [25,43]. Strains vary in ability on the MWM, which can relate to variation in anxiety or visual acuity [25,44]. We used C57BL/6J×SJL hybrid mice. One advantage of using hybrid strains is that recessive mutations that might confound interpretation show less penetrance [45]. C57BL/6 mice competently learn the MWM but SJL mice (that may express visual impairment) do not [25]. Visual acuity of our mice was previously confirmed [36,46], and all mice employed showed intact visual responses.

Our previous results with an eight-choice cued maze documented that transgenic growth hormone mice had remarkably superior learning in youth but by about 1 year of age could not learn the task [36]. If supplemented, however, transgenic mice showed no declines but instead learned significantly faster as they aged [36]. Thus, the supplement was highly effective in a model of rapid brain deterioration resembling AD. Results presented here importantly attest that the DSP also strongly offsets normal brain aging.

 Fifty percent of all untreated mice showed no evidence of learning even after 15 trials over 5 days compared to only two out of 17 mice in the supplemented group (chi-square: p<0.05). In our previous studies with an eight-choice cued maze, 33% of normal mice (all untreated) also showed no evidence of learning particularly when older. Learning of both tasks tends to be an all-or-nothing phenomenon, mice either expressing steady incremental improvement or little progress [36,46,47]. It is all the more remarkable then that older supplemented mice effectively learned the MWM, achieving a 46% reduction in latency to reach the hidden platform by day 5 (Fig. 1). It appears that the DSP does not simply improve cognition but actually enables learning in a large proportion of mice that might otherwise not learn. Moreover, the threshold nature of this learning suggests that higher-order processes associated with “insight” may be favored by the supplement.
Cognitive decline is one of the most reliable biomarkers of aging in animals [28,48]. Thus, prevention of cognitive decline in normal animals spanning ages of 12 to 27 months attests to great efficacy of the supplement (Fig. 1).

We recently documented that our DSP prevented declines in spontaneous motor activity that, like cognition, is regarded as a fundamental biomarker of aging [30]. This raises the possibility that supplementation could change swimming speed which can confound results for the MWM [44]. Mean swimming speed, however, proved virtually identical on either treatment. Given that exercise can benefit cognitive function [49,50] and spatial learning [i.e., MWM: 14], however, increased spontaneous activity could synergize with the DSP to maintain youthful cognition into advanced ages.

**Mitochondrial function:** Maintaining effective mitochondrial function was a specific design criterion for our DSP (e.g., α-lipoic acid, acetyl-L-carnitine, B vitamins, coenzyme Q10, maintenance of mitochondrial membranes, and general antioxidant protection). Strong alterations in mitochondrial function indicate that effective dietary components penetrate the blood-brain barrier and impact mitochondria (key criteria sought for anti-aging interventions). We previously reported that untreated mice experienced 46% loss of complex III activity in brain by 2 years of age whereas mice supplemented with the DSP showed a 56% increase [30]. Two-year-old mice had 85% greater complex III activity if supplemented [30]. Here we document a similar age-related pattern for complex IV. Untreated mice showed 40–50% loss of complex IV activity between 3 and 24 months of age (Fig. 2). In contrast, supplemented mice expressed a 65% gain in complex IV activity between 3 and 24 months (Fig. 2). Nutrients altering mitochondrial activity can impact numerous brain functions by modulating ATP availability [51]. We know of no other intervention so strongly impacting mitochondrial activity.

Activity of mitochondrial ETC complexes and ATP production generally decline with age [29], and declining complex IV activity is a particularly reliable biomarker of aging associated with cognitive loss [29]. Neurodegenerative conditions (e.g., AD) are also characterized by mitochondrial dysfunction and declining complex IV activity [34,52]. Selective reduction of complex IV in rat primary hippocampal neurons results in neuronal
death [53]. Whether AD is a specific pathology or simply an extreme manifestation of normal senescence remains debatable [4]. Although previous aging theory highlighted free radical damage as a primary cause, mitochondrial dysfunction and energy shortfalls may contribute to both normal and pathological cognitive declines. Indeed, learning and activity of ETC complexes (III, IV, and III+IV) were positively correlated in mice more than 1 year old (Table 1). Hence, it appears that mitochondrial function is linked to cognitive capacity in old age.

We found no correlation between activity of either complex III or IV and learning of the MWM in young animals (Table 1). Supplemented mice actually showed lower levels of complex IV (Fig. 2) and complex III [30] activity in youth although this did not compromise cognition (see: Fig. 1) or levels of physical activity [30].

**Brain size:** Brain size is positively associated with intelligence in humans [54], and growth hormone transgenic mice that have larger brains also had vastly superior learning on an eight-choice visually cued maze and novel object recognition and memory tests [36,47]. Rodents selected for larger brains (and associated increases in body size) also display enhanced learning [55]. Early studies noted great individual differences with respect to aging of human brains [56] suggesting interventions are possible. Reduction in size of most major brain regions occurs in aging resulting in significantly reduced brain mass [57,58].

Both our supplemented and untreated mice showed diminishing mass-specific brain weight with age, particularly in females (that also had larger relative brain size than males in youth) (Fig. 3). Supplemented mice maintained larger brains than controls throughout life and in old age brains of supplemented mice were 7% larger in males (p<0.02) and 11% larger in females (p<0.02) (Fig. 3). Hippocampal function critical to memory is exceptionally sensitive to aging [1,59]. The MWM test is considered to test hippocampal function [60]. Neurodegeneration of the hippocampus is widely implicated in the loss of spatial memory (navigation skills), dementia, and AD [21,59]. In AD, the rate of hippocampal atrophy is 3.5–4%/year, exceeding the ∼1.6%/year loss observed in normal aging by nearly 3-fold [61,62]. For normal aging and neurodegenerative
conditions alike, diminishing hippocampal volume is correlated with atrophy of other brain regions, increasing ventricular space and diminished brain volume [59,63]. Consequently, brain size is a strong predictor of cognitive decline, hippocampal degeneration, and risk of AD [63,64]. It is likely that the DSP benefited aging hippocampal function.

**Complex dietary supplements:** Esposito et al [65] reviewed literature on antioxidant treatment of various neurodegenerative diseases. Of 13 materials mentioned, our DSP contains eight. In a compilation of nutrients positively impacting cognitive functions, 13 of those identified are present in our DSP [51]. A study providing omega 3 fatty acids combined with iron, zinc, folate, and vitamins A, B6, B12, and C to children (five of these are in our DSP) obtained improved scores on tests of verbal intelligence, learning, and memory [66]. Such results were considered indicative of synergism among ingredients [51]. A meta-analysis of studies administering supplements to children that contained at least three ingredients found a marginally significant improvement in fluid intelligence (reasoning) and a significant improvement in academic performance [67].

Zahs and Ashe [68] compiled a list of interventions that positively impact AD symptoms in APP mouse models. Fourteen of the dietary interventions listed are in our DSP. Human subjects with mild AD showed improvement in the delayed verbal recall task after just 12 weeks on a complex dietary supplement which shares 14 ingredients with our DSP [69]. Of 20 materials considered of possible value in Alzheimer’s in an authoritative review, 12 are in our DSP [70]. Several reviews have suggested that multiple supplements promise to yield the greatest benefits [e.g., 65,69]. Our results demonstrate proof of this principle.

Diets high in fruit and vegetables also positively impact brain aging and behavior, but none shows the magnitude of benefits we have documented in this and previous papers [71,72]. The particular value of specific plants or dietary mixes of fruit and vegetables not only reflect specific content (e.g., flavonoids) but likely involves interactions and additive effects. Most ingredients in our DSP occur naturally in foods and spices. Unlike normal meals, the DSP combines ingredients specifically targeted to five mechanisms of
aging and provides these in relatively concentrated form.

**Conclusion:** Despite generally disappointing results in studies applying one or a few dietary supplements, our results show that complex supplements can powerfully impact brain aging in normal animals as well as neuropathology in a transgenic mouse. The DSP had a relatively broad spectrum of actions: It augmented mitochondrial function, increased mass-specific brain weight, and markedly improved cognitive abilities in old age. Our previous studies demonstrated that it elevated expression of brain neurotransmitter systems and ameliorated age-related motor decline [30]. Such effects likely emerge from additive, synergistic, and recycling interactions among ingredients and the fact that the DSP was designed to ameliorate multiple mechanisms of aging. Since materials in DSP are available in health food stores, a large sector of the aging human population could obtain nearly immediate access should human trials confirm safety and benefits.

**2.5 ACKNOWLEDGMENTS**

This research was supported by a grant to CDR from the Natural Sciences and Engineering Research Council of Canada. We thank Zoya Tov for her contributions to diet preparation and technical support for the experiments.
2.6 FIGURES and TABLES

**Table 1.** Correlation analysis between learning performance in MWM (day 5 latency to locate hidden platform) and activity levels of brain mitochondrial complexes in younger (<1 year old) and older (>1 year old) mice

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†Mitochondrial activity for each mouse was predicted from their age using equations relating mitochondrial activity to age for supplemented or untreated animals.
Figure 1. Latency to locate the escape platform in the MWM for untreated (n=20) and supplemented (n=17) young mice (<1 year) and old (>1 year) mice. No gender differences were resolved so genders were pooled. Both treatment groups had high latencies on day 1 irrespective of age. On day 5, young mice from both treatment groups showed nearly identical learning (~43% improvement) so treatments were pooled to improve resolution (p<0.02). Old untreated mice did not show any noticeable improvement by day 5 (a difference of 8% was not significantly resolved). However, old supplemented mice expressed a level of learning virtually identical to young mice (46% improvement on day 5 compared to day 1; *p<0.05) and a 47% reduction in latency compared to old untreated mice (*p <0.05)
Figure 2. Age-related patterns of complex IV activity in brain mitochondria for untreated (n=9) and supplemented male (n=9) mice. Slopes of age-related linear regressions differ significantly between supplemented and untreated mice (ANCOVA, covariate=age, slopes differ: $p<0.02$) indicating that opposite age-related trends between treatments were statistically resolved. Linear regressions: Untreated: $r^2=0.272$; $p=0.150$; $y=26.303-0.527\times x$; Supplemented: $r^2=0.437$; $p=0.052$; $y=10.835+0.665\times x$
Figure 3. Mass-specific brain weight of untreated (n=73) and supplemented (n=32) male and untreated (n=42) and supplemented (n=35) female mice with respect to age. Age-related regressions for brain weight of male mice were not significant: untreated males: $r^2=0.043; \ p=0.081; \ y=15.247–0.056*x$; supplemented males: $r^2=0.035; \ p=0.308; \ y=16.238–0.061*x$. Mean mass-specific brain weight of supplemented males was 7% greater compared to untreated controls (t-test: $p<0.02$). Age-related regressions for brain weight of female mice were negative and significant for both treatment groups: untreated females: $r^2=0.239; \ p<0.001; \ y=20.405–0.240*x$; supplemented females: $r^2=0.290; \ p<0.001; \ y=21.814–0.215*x$. Mean mass-specific brain weight of supplemented females was 11% greater compared to untreated controls (t-test: $p<0.02$). Since brain weight was significantly correlated with age in females, ANCOVA (covariate=age) was also applied and returned a significant difference ($p<0.001$) between supplemented and untreated females. Mean mass-specific brains weight of female mice was 18% greater compared to male mice (t-test: $p<0.0001$)
2.7 REFERENCES


[53] Atamma H, Killilea DW, Killilea AN, Ames BN. Heme deficiency may be a factor in the mitochondrial and neuronal decay of aging. PNAS. 99: 14807–14812, 2002


2.8 APPENDIX

Figure A1. Representative paths taken by an old untreated (A) and an old supplemented mouse on day 1; from release point (white square) to hidden platform. Paths visualized by Noldus® EthoVision image tracking software. Untreated old mice showed lack of learning as evident from random swim patterns on day 5 (example: C). Supplemented mice learned the location of the hidden platform by day 5 and were able to reach it via a more direct route (example: D). Paths clearly indicate that our mice did not employ a chaining search strategy.
CHAPTER 3

Chapter introduction:

Similar to motor and cognitive function, sensory decline is common in aging animals. Irreversible age-related vision loss in humans is primarily attributed to age-related macular degeneration (AMD). Olfactory deficits are linked to neuronal deterioration in the olfactory bulb preceding onset of neurodegenerative diseases such as Alzheimer’s and Parkinson’s. Effects of the DSP on olfactory sensitivity and visual acuity in old mice are presented in conjunction with histological analyses of the olfactory bulb and retina of the eye. Results have strong implications for prevention of AMD and neurodegenerative pathologies.

Motor coordination and motor learning are mainly attributed to the cerebellum. Loss of vulnerable cerebellar neurons (Purkinje cells) may begin in early to mid-adulthood further exacerbated in later years. Loss of Purkinje neurons was offset by the DSP and reflected by improved performance on a test of motor balance. The cerebellum also participates in navigation and spatial memory, linking the current findings with previously reported spatial learning improvement [Ch. 2].

Histological assessment (cell counts and structural morphology) of brain regions implicated in aging importantly contributes to my work by providing direct evidence in support of DSP-mediated neuroprotection. Additional evidence is presented in Chapter 6 and the possibility of enhanced neurogenesis is also considered.

Before describing the aforementioned results, this chapter addresses impacts of the DSP on emotionality. Somewhat unexpectedly, we observed anxiolytic effects of supplementation in normal mice. Aging is not a significant risk factor for anxiety disorders; however, elevated anxiety may have greater consequences in older persons. Regardless, possible therapeutic applications for humans emerge.

In addition, the link between anxiety and motor balance disorders suggests shared neurocircuitry, which is explored in this chapter. A thorough synthetic discussion of anxiety and cognition with respect to shared mechanisms targeted by our DSP is included in a supplementary discussion section.

Note: Citation number [11] refers to Chapter 1; and citation number [12] refers to Chapter 2
CHAPTER 3

TITLE

A Complex Dietary Supplement Augments Sensory and Behavioral Function, Modulates Anxiety and Prevents Neuronal Atrophy in Aging Mice

AUTHORS

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3.1 INTRODUCTION

Aging is associated with declining cognitive, sensory and motor functions [1–16]. Active and social lifestyles as well as balanced diets can have considerable impacts on rates of functional aging [17–22]. This suggests that humans (and other organisms) may retain some capacity for expressing youthful functional phenotypes in advanced ages. Moreover, sizable improvements appear attainable through relatively undemanding interventions.

In previous work, we showed that age-related cognitive and motor declines could be prevented or delayed by dietary supplementation. We employed a complex dietary supplement (DSP) designed to specifically target five critical processes associated with aging [10–12,23]. Even in advanced ages, supplemented mice exhibited youthful cognitive and motor abilities [10–12]. While intensity of physical activity was reduced with age [Ch. 4], the duration of daily locomotion was unchanged from youth well into oldest ages [11]. Thus, mice indeed retain the capacity to achieve youthful cognitive functionality, and to a large degree, youthful motor capabilities into advanced ages.
Limitations particularly applied to intense physical activity suggest peripheral constraints as opposed to central regulatory limitations. Improved cognition and physical abilities in supplemented animals were attributed to reduced oxidative and nitrative damage and enhanced mitochondrial activity [11–13]. A wider realm of cellular biomarkers is being investigated.

In the present work, we examined whether benefits of the DSP extended to sensory and behavioral function in aging mice. Mice were subjected to a battery of tests to assess motor coordination, vision, olfaction, emotionality and contextual discrimination.Remarkably, supplemented mice showed sizable improvements in virtually all aspects examined.

For the elderly population, the advantages of maintaining intact cognition and physical abilities extend well beyond quality of life and independence. In previous works we described strong associations between cognitive and physical decline with risks of developing severe disabilities and mortality [see: 11–13; Ch. 5–7]. However, age-related functional loss is not limited to cognition and physical activity.

Aging is associated with rapid deterioration of sensory and somatosensory functions including vision, olfaction, and motor coordination [1–3,5–8]. While not inherently fatal these conditions are associated with substantially elevated morbidity and mortality [24–33], and obvious impacts on quality of life. Age-related deterioration of motor coordination is a major cause of falls in the elderly, exacerbated by declining balance and physical strength [24]. Falls are a leading cause of disability and deaths in persons over 65 years of age in the US [24].

Irreversible vision loss in elderly persons is primarily attributed to age-related macular deterioration (AMD) [34,35]. This presently affects 6.5% of Americans and is projected to double over the next ten years [34]. AMD is associated with a five-fold increase in cardiovascular complications and mortality [32].

Olfaction is of unique clinical importance, as it is now well established that olfactory deficits precede and accurately predict onset of debilitating neurodegenerative conditions such as dementia, Alzheimer’s and Parkinson’s [25–31,33]. In addition, loss of olfaction
may have further health risks associated with detection of spoiled foods and volatile toxic agents [7,8].

Emotional disorders are common in the elderly, however aging alone does not appear to be a significant risk factor [36]. Somewhat unexpected anxiolytic effects of our DSP prompted a closer investigation into regulatory modulation of anxiety, which may share common mechanisms with aging.

Appropriately manifested anxiety is adaptive for avoiding or coping with potential environmental threats. However, chronically elevated anxiety can lead to avoidance and unsubstantiated fear of situations in absence or real danger [37,38]. Roughly 30% of Americans suffer from anxiety disorders in their lifetime [39] which is associated with increased risk of cardiac morbidity and mortality [40,41]. Anxiety disorders can impair workplace performance effecting quality of life and incurring economic costs [42].

Particular consideration was given to the apparent anxiolytic effects of our DSP because anxiety behaviors and cognition (specifically memory formation and learning) show extensive neurochemical overlap. Also, a link between anxiety and motor balance disorders is established [43,44] implying shared neurocircuitry. Finally, some evidence suggests that elevated anxiety can be a pre-clinical symptom of neurodegenerative conditions, such as Parkinson’s disease [45].

Neurodegeneration is strongly exacerbated in aging and contributes directly to cognitive, motor and sensory impairments [9]. Aging renders neurons vulnerable to degeneration; yet considerable individual variability in aging rates and different susceptibility of particular neural systems to aging suggest room for intervention [9]. The cognitive and motor improvements obtained by the DSP [10–12] point to possible neuroprotective effects. Further, increased brain size in supplemented animals [12] supports the likelihood of higher neuronal number and synaptic connections.

Presently, we document that our DSP prevented age-related loss of neurons in two key regions of the brain, the cerebellum and the olfactory bulb. Morphological assessment also suggested that neuronal atrophy was ameliorated in these regions. Behavioral studies showed augmented sensory and motor function consistent with increased neuronal
populations in brains of old supplemented animals. Additionally, histological examination of retinal structure revealed markers indicative of higher numbers of photoreceptor cells in aging supplemented mice. This correlated with better visual acuity, and suggests that impacts of DSP are not limited to neurons, but may extend to other cell types and cellular aging more generally.

This research links behavioral aspects to biomarkers in relevant brain regions and peripheral measures as impacted by the DSP. A literature synthesis integrates new and current understanding of aging with respect to behavioral, physiological and anatomical features presently examined. Cellular, molecular and systemic dysregulations accompanying aging are revisited and possible mechanisms of our dietary intervention are proposed in line with improved functionality and neuroprotection. Earlier findings are briefly re-examined in light of current results.

3.2 METHODS

Animals: Test animals were normal (Nr) and growth hormone transgenic mice (Tg) of C57BL/6×SJL hybrid background. Tg mice express accelerated aging and a variety of amplified progeroid aging features. All husbandry protocols adhered to Canadian Council for Animal Care guidelines. Mice were housed in standard plastic cages with rodent chow (Teklad® 8640) and water provided ad libitum. Animal rooms were maintained at 23±2°C and 12/12 hour light/dark photoperiod. Genders, genotypes, ages and numbers of mice used in various studies are indicated as appropriate.

Complex dietary supplementation: We designed a complex dietary supplement (DSP) including 30 nutraceutical ingredients targeting five key mechanisms associated with aging. Composition of the DSP and protocols for preparation and administration were previously described [10,11]. Biological actions of individual components are summarized in [23]. At birth, mice were randomly assigned to either the supplemented or untreated group. Supplemented mice were treated daily from weaning and for life. Complex dietary supplementation can impact multiple targets and allows for potentially
strong synergetic and co-stimulatory interactions not possible for formulations employing one or a few ingredients.

Somatosensory tests: All mice used in these tests were Nr ~2 year old males and females.

Landing response: Mice were held by the base of the tail and lowered onto a flat surface short of contact. Mice with intact vestibular function extend their forepaws in anticipation of landing.

Negative geotaxis: Similar to the landing response, this test assesses vestibular function [46]. Mice were placed on a 30° inclined plane facing down. Mice with uncompromised vestibular function quickly orient their body position to face upward.

Visual placing: This test was previously used to grossly assess vision [47]. Mice held by the base of the tail were lowered past a black table edge, far enough to prevent vibrissae contact. Mice with intact vision reach for the table edge with their front paws.

Pinch reflex: Mice were lightly pinched by the hind paw. Animals with normal pain sensation and reflexes immediately withdraw the paw.

Behavioral experiments: Open field: The bright open field test was previously applied [47] to assess emotionality. A square arena 70×70×45cm (W×L×H) constructed from white Plexiglas was illuminated by two 100W white lights. A 50×50cm square in the middle and the 10cm wide outside border constituted ‘central’ and ‘peripheral’ zones, respectively. Mice were placed singly in the central zone and videotaped from above for five minutes. Arena was cleaned with ethanol between runs. Image tracking software (Noldus® EthoVision) was used to score variables: (a) latency to exit central zone, (b) distance traveled in central zone (c) distance traveled in peripheral zone, and (d) mean running velocity.

Step-down test: Mice were individually placed on top of a circular platform (10cm d × 7cm high). First, trials were conducted in bright illumination (two 100W white lights). On a later date this test was repeated in dim lighting (single 25W red light). Latency to step down was recorded (ten minute cut-off).
Circle run: This test was developed in our lab and previously used to assess emotionality in mice [47]. A circular white arena 1 m in diameter was surrounded by a shaded overhang 5cm high and 20cm wide, providing a potential dark refuge. An open-top cup (7cm d × 5cm deep) was fitted in a depression in the center of the arena such that the top of the cup was level with the arena floor (see: Fig. A1 in Appendix). A mouse was placed in the cup and videotaped from above. Latency to climb out of cup (four paws) followed by latency to reach the shaded overhang were scored. Mice were tested on two separate trials two weeks apart, first in bright, and then in dim illumination. Bright open areas are naturally aversive to mice and elicit anxiety-like behavior [47]. Conversely, dim illumination is less aversive allowing for observation of active or exploratory behavior [48].

Rotarod: This standard test assesses motor coordination and balance [49]. Mice were individually placed on a horizontal plastic cylinder 6cm in diameter rotating at 12rev/min. [see: 49] Latency to fall onto a soft landing pad was scored on three consecutive trials. Improvement was assessed by subtracting latency to fall on first trial from the latency on the last trial.

Visual acuity: Based on the principle underlying the visual placing test, we developed a more precise assay for measuring visual acuity. Instead of a single visual cue, this test uses an array of increasingly more challenging visual cues. Mice were suspended by the base of the tail and repeatedly lowered past a black horizontal wire at a distance of 5cm (to avoid vibrissae contact). If able to see the wire mice reached towards it with the forepaws. Lack of a reaching response on five consecutive attempts indicated that mice were unable to see the wire. The next set of challenges consisted of varying wire thickness, background contrast (i.e. white or black background) and room lighting. Specifically, mice were challenged with: (a) 5mm wire suspended over white background, (b) 5mm wire suspended over black background, (c) 1mm wire suspended over black background, (d) 0.5mm wire suspended over black background, (e) 0.5mm wire suspended over black background in dim light, and (f) 0.5mm wire suspended over
black background in near darkness. Mice were tested in order of least to most challenging test with 30min inter-trial intervals.

**Olfactory sensitivity:** Test protocols were adopted from Witt et al [50]. Three serial dilutions were prepared by diluting 1.25, 2.50 and 5.00mg of peanut butter in mineral oil to a final volume of 100ml. A fourth blank dilution (mineral oil only) was used as a negative control. Mice were individually placed in a 28×16×12cm plastic enclosure and acclimated for 30 min. A camera was set up to record behavior. Just prior to starting a trial, 1ml of a peanut butter dilution was pipetted onto a 3×3cm filter paper square. The filter paper was sealed inside a small Petri dish with several round holes to allow dispersal of scent and placed in the enclosure. Each mouse was tested on all four peanut butter dilutions in a random order with 2–3 days in between trials. Olfactory sensitivity was assessed by scoring the time spent exploring the Petri dish (e.g. sniffing, licking, biting) in a 10 minute time interval.

**Tissue histology:** In mid-photophase, mice were decapitated and brains and eyes were removed. Cerebellum and olfactory bulbs were separated. All tissues were placed in 10% formalin solution, processed overnight and embedded onto paraffin blocks. Tissues were sliced at 5 microns on a microtome and stained with hematoxylin and eosin (H&E) and Nissl stain. Mice aged 14–17 months, n=6 untreated and n=6 supplemented.

**Tissue morphology and cell counts:** **Cerebellum:** Thickness of molecular layer and granule cell layer was assessed in lobules II and III using ImageJ software (see: Fig. 5 caption). Number of Purkinje cells in lobules II and III were counted. Some studies estimated total cerebellar cellularity by interpolation of counts from an array of sections spanning the entire width of the cerebellum [51]. However, we were only interested in the relative difference between treatment groups, hence a single representative inter-hemispheric sagittal section was sufficient [52].

**Retina:** Mid-sagittal sections of eye were used to measure the thickness of the outer nuclear layer and the outer fragment layer in retina. As age-related loss of photoreceptors is most prominent in the central retinal portions; therefore measurements were done on
retinal cross-sections 1000µm inferior and superior to the position of optical nerve attachment (see: Fig. 7 caption).

Olfactory bulb: One olfactory bulb from each animal was randomly chosen. Thickness of the glomerular layer, external plexiform layer as well as counts of mitral cells were performed (described in: Fig. 10 caption). Morphology and organization of mitral cells and techniques for assessing cell number are summarized in Appendix.

Statistical analyses: Statistical analyses were performed using Statistica® 6.0 software. Statistical tests applied are described in corresponding figure and table captions. Briefly, where age-ranges were available, effects of treatment, genotype, gender and other independent variables were first assessed with ANCOVA (covariate=age). If main effects or interaction effects were not resolved for a given predictor variable, groups (or ages) were pooled. Effects of the remaining variables were discriminated with post-hoc SNK or Duncan’s tests. In the absence of other predictor variables, effects of treatment were resolved with a t-test. A two-tailed (Fisher’s) chi-square test was used when comparing number of animals eliciting positive responses in each treatment group on behavioral tests of visual acuity and somatosensory function. Where applicable, age-related effects were analyzed with linear regressions.

3.3 RESULTS

Open Field: Nr and Tg mice aged between 4–18 months were scored on four parameters in a brightly illuminated open field (n=56).

(a) Latency to exit the central zone was not affected by age, genotype or treatment (data not shown).

(b) Distance traveled in peripheral zone was affected by age and genotype but not treatment (probabilities in Table 1). Oldest mice (15–18 months) traveled ~15% less (data not shown), and Tg covered ~40% less distance than Nr mice (Fig. 1A). Slightly greater distances traveled by supplemented mice were not significantly resolved (Fig. 1A). All mice traveled greater distances in peripheral compared central zone (Fig. 1A).
(c) Distance traveled in central zone was reduced across age-range (~30% from 4 to 18 months; probabilities in Table 1). A significant effect of genotype was resolved (Table 1) but this reflected a genotype*treatment interaction mainly associated with changes in Nr behavior. Nr supplemented mice traveled more than double the distance covered by untreated Nr (p=0.003; Fig. 1A). Supplementation had no effect on distance traveled in the central zone in Tg mice (Fig 1A).

(d) Mean running velocity was not affected by treatment (Table 1). Tg were slower compared to Nr mice by 24% and mean running velocity was negatively correlated with age (all probabilities in: Table 1).

Parameters (a–d) showed significant age-related regressions when genotypes and treatments were pooled (Table 1). For each parameter we assessed whether supplemented and untreated Nr and Tg mice resolved different slopes for age-related regressions. Differences in slopes were not resolved with ANCOVA (Table 1) indicating that impacts of age were similar for all groups and were unaffected by genotype or treatment.

Step-down test: Latency to step down from an elevated platform was first measured under bright illumination, and at a later date, in near-darkness. Some animals used in the dark trails were different than those tested in bright illumination. Latency to step down was significantly affected by treatment and illumination, and only marginally by age and genotype (Table 2). Therefore, to increase statistical power, ages and genotypes were pooled. For untreated animals, the latency to step down did not significantly differ between bright (n=67) and dark (n=39) illumination (Fig. 2). However, in supplemented mice differences between bright (n=73) and dark (n=21) illumination resulted in a significant 38% decrease in step-down latency (Fig. 2). In bright conditions, supplemented mice showed a marginal (p=0.151) decrease in latency compared to untreated controls. But in dark illumination, latency to step down was significantly reduced in supplemented mice (p=0.038; Fig. 2).

Circle run: Effects of age, genotype, treatment and illumination on latency to (a) emerge from cup and (b) escape to shade are presented in Table 3. The same group of mice was tested in bright and dark conditions one week apart. Effects of age and
genotype were not strongly resolved (Table 3); hence Nr and Tg mice were combined across ages.

(a) Latency to emerge from cup: Untreated mice took an identical time to emerge from the cup regardless of illumination (Fig. 3). Compared to untreated mice, supplemented mice took 34% longer (p<0.0001) to emerge in bright illumination (Fig. 3). Alternatively, when tested in near-darkness, supplemented mice emerged from the cup significantly faster (p<0.0001) than in bright lighting (Fig. 3).

(b) Latency to escape to shade: Again, the latency to escape to shade was unchanged in untreated mice despite a change in illumination (Fig. 3). In bright conditions, supplemented mice spent 17% (p=0.048) longer in the open zone before entering the escape overhang compared to controls (i.e. less anxiety). However, when tested in darkness, escape time of supplemented mice was actually 18% faster compared to controls (p=0.019; Fig. 3). This opposite effect is also reflected in the significantly different escape latency of supplemented mice going from bright to dark illumination (p=0.006; Fig. 3).

Rotarod: Latency to fall off a rotating cylinder was measured on three consecutive trials. Mean (3–trial average) latency was nearly 4 times lower in Tg compared to Nr mice; but effects of treatment on mean latency were unresolved in either genotype (Fig. 4A). A key aspect of this test, however, is rate of improvement. Improvement was scored by subtracting latency to fall on the first trial from that on the last attempt. Improvement was not affected by genotype (p=0.390); hence we combined Nr and Tg mice for better statistical power (Fig. 4B). Untreated mice showed no improvement between by the last trial, but supplemented mice improved by ~40 seconds, which was significantly resolved (p=0.044; Fig. 4B). This improvement corresponded to one third of the initial latency.

Cerebellum histology: Purkinje cells (PC) in inter-hemispheric sagittal cerebellar sections of lobules II and III (Fig. 5A) were counted in Nr mice aged 14–17 months (narrow age-range precluded analysis of age-related trends; supplemented: n=6; untreated: n=6). PC are restricted to the Purkinje cell layer (Fig. 5B) and length of the Purkinje cell line must be accounted for when counting cells [51,52]. Supplemented mice
showed a 20% increase in PC number per mm of cell line (t-test: p<0.05; Fig. 6A). Figure 5D shows a close up of PC from supplemented and untreated mice dyed with Nissl stain. PC in a sample from an untreated mouse show visibly reduced PC number compared to a supplemented mouse (Fig. 5D).

Average thickness of the molecular layer (ML) and granular layer (GL) in lobules II and III were calculated using ImageJ software according to the schematic shown in Figure 5C. Supplemented mice had a 16% and 18% increase in thickness of the ML and GL, respectively, compared to controls (t-test: p<0.05; p<0.01; Figs. 6B and C).

Cerebella were collected from three male and three female mice in each treatment group. A main effect of sex or sex*treatment interactive effects were not resolved.

**Simple somatosensory tests:** Results of the (a) landing response, (b) visual placing, (c) negative geotaxis, and (d) pinch reflex are summarized for Nr (~2 y old) mice (Table 4). Virtually all mice showed normal function in these simple somatosensory tests and further improvement from supplementation was not possible.

**Visual acuity:** A newly developed protocol was used to assess visual acuity of 1.5–2 year old Nr mice (see: Methods). Animals were challenged with six visual cues of increasing difficulty. A reaching response indicated that mice were able to see the cue. All mice showed a reaching response on the least difficult challenge (#1; Table 5). As difficulty increased (challenges: #2–5), fewer mice successfully located the visual cue. The number of animals maintaining positive responses was invariably greater in the supplemented group (Table 5). On challenge #4, 63% of supplemented mice expressed reaching compared to only 23% of untreated mice (chi-square: p=0.014; Table 5). In fact, a steep (>60%) drop-off in reaching responses by untreated mice occurred between challenge #3 to #4. In supplemented mice, a comparable drop was only seen between challenges #4 to #5. Thus the DSP elevated visual acuity. No mice showed reaching responses on challenge #6 (Table 5) which employed nearly complete darkness. Darkness provided a sort of negative control reinforcing that responses in other challenges were indeed due to visual aspects.
Thickness of outer nuclear layer (ONL) and outer segments (OS): Mid-sagittal sections of eyes were stained with H&E in supplemented (n=4) and untreated (n=4) 2 year old Nr females (diagram: Fig. 7A). In supplemented mice, thickness of ONL and OS averaged over the length of proximal retinal sections (see: Fig. 7 caption) were 26% and 29% significantly greater, respectively, compared to controls (p<0.05; Fig. 8). Close-ups of retinal sections from supplemented and control mice showed visible differences (Figs. 7B–E).

Olfactory sensitivity: Olfactory sensitivity was measured by quantifying the duration of exploration in response to varying concentrations of an attractive scent (peanut butter). Both supplemented and untreated mice spent little time (~130s) investigating filter papers with zero or low (0.125mg/10ml) concentrations of peanut butter. When presented with higher (0.250mg/10ml and 0.500mg/10ml) concentrations, supplemented mice increased exploratory duration by 68% and 105%, respectively. Plotting the mean duration of exploratory behavior (y-axis) over peanut butter dilution (x-axis) for supplemented mice returned a strong positive correlation (i.e. dose response) ($r^2=0.976; p=0.012; $ Fig. 9). Conversely, untreated mice showed nearly no change (<10% increase) in exploration going from zero to high peanut butter concentrations ($r^2=0.069; p=0.738; $ Fig. 9). Slopes of regressions significantly differed for treatments (ANCOVA: $p=0.036$).

Olfactory bulb histology: Cross-sections of olfactory bulbs were obtained from supplemented (n=6) and untreated (n=5) mice (ages: 14–17 months). ImageJ was used to calculate mean thickness of glomerular layer (GLM) and external plexiform layer (EPL) (organization of neuronal layers shown in: Fig. 10A). GLM and EPL were significantly reduced in supplemented mice by 25% and 28% respectively, compared to controls (t-test: $p=0.026; p<0.001; $ Figs. 11B and C).

Mitral cell layer: For counts of mitral cells (see: Appendix) we applied strict criteria including cell size, shape, position, and visibility of clearly defined nuclear envelope and nucleolus (see: Figs. 10B and C and figure caption). Although this underestimates total cell numbers it provides maximal accuracy for comparisons between samples. Mitral cell counts in a 1mm representative section were 29% higher in supplemented mice compared
to controls (t-test: p=0.030; Fig. 11A). Despite this significant resolution, greater samples numbers, more representative sections and additional staining techniques could reinforce these preliminary findings.

3.4 DISCUSSION

**Emotionality:** Variants of the open field test are used to assess emotionality in rodents [47,48,53–56]. A simple step-down test also provides a clear score related to anxiety [47]. However, depending on protocol variations and parameters scored, results may be more indicative of exploratory behavior than anxiety-driven responses [48,55,57]. Multiple tests and careful considerations of settings are required to assure correct inference [55]. Variables measured in tests for anxiety (e.g. open field, elevated maze) were more consistent with general activity levels when carried out in dim light when mice are normally active [48]. To assess emotionality we employ bright illumination and white Plexiglas construction creating aversive environments. Previously, we showed that such protocols elicited anxiety-like behaviors rather than exploration [47].

Collectively, tests of emotionality (Figs. 1–3) suggest that the DSP reduced anxiety in mice. In the open field test, supplemented Nr mice showed less restricted movement in the central zone compared to untreated Nr controls (Fig. 1). This suggests that anxiety thresholds were elevated by DSP allowing mice to be less emotional towards exploring an ‘unsafe/novel’ environment. Analogous findings were obtained using the circle run test. This design combined the open field test with light/dark preference [47]. In bright illumination, untreated mice emerged faster from the cup and ran towards the shaded overhang (Fig. 2). Supplemented mice took longer in comparison indicating reduced anxiety associated with bright open areas. The step-down test carried out in bright illumination supported impacts of the DSP on emotionality, although effects of treatment were only marginally resolved (Fig. 3) due to high individual variability.

While the DSP was not intentionally designed to target emotionality, the present findings indicate that it indeed has significant anxiolytic properties.
**Confounding effects of physical activity:** Essentially all variants of behavioral anxiety tests rely on movement of animals to infer emotionality levels [47,48,55]. Therefore, intrinsic differences in activity levels between treatment groups (if present) may bias interpretation. Our DSP was previously shown to augment physical activity [11]. This could influence movement of animals in the experimental apparatus irrespective or emotionality. However, in the *open field test*, distance traveled by mice in the peripheral zone (Fig. 1) and mean running velocity (Table 1) were unaffected by treatment. This indicates that increased exploration of the central zone by supplemented Nr mice was not a consequence of upregulated activity. In the *circle run test*, increased physical activity of supplemented mice would predict faster escape; however, the opposite result was observed (Fig. 2). This supports that differences in intrinsic activity levels between treatment groups did not confound results.

**Anxiety disorders:** Inappropriately manifested or chronic anxiety leads to unsubstantiated fear and avoidance of places in absence of real danger [37,38]. Anxiety disorders are diagnosed in nearly a third of Americans [39] and are associated with increased risk of morbidity and mortality [40,41]. Generalized anxiety disorders share common etiology with obsessive-compulsive disorder (OCD) [58], a disabling condition affecting over three million Americans [59]. OCD is also associated with impaired visual memory in humans [60] suggesting that cognition and anxiety share neurocircuitry. Indeed, pharmacological or genetic manipulations causing anxiety also impact memory and cognition [61–64]. Our DSP both improved cognitive function [10,12] and ameliorated anxiety (Figs. 1–3) in aging mice, suggesting shared underlying mechanisms. Regardless, the present study holds promise that the DSP may benefit human conditions such as OCD and other syndromes associated with anxiety, phobias and panic disorders [37].

**Mechanisms of anxiety in aging:** Emotional behavior can increase with age in mice [57]; although many strains show opposite trends [65]. Aging alone is not a strong risk factor for emotional and anxiety disorders in humans [36]. Yet, some mechanisms associated with emotionality involve pathways implicated in aging.
Oxidative stress was linked to elevated anxiety in aging rodents [57,66] and reactive oxygen species (ROS) in brain positively correlate with anxiety behavior [53,67]. Both ROS [68] and inflammatory processes [69,70] are strongly implicated in aging and age-related diseases. Furthermore, ROS and inflammation appear to be co-stimulatory [71–74]. Elevated ROS via NADPH oxidase activity induced anxiety which was reversed by apocynin (an NADPH oxidase blocker) [75]. In addition, chronic inflammation and inflammatory cytokines were shown to induce anxiety in mice and rats [76,77]. There are multiple mechanisms linking ROS and cytokines to modulation of anxiety. Neuronal losses in stress-regulating regions of the brain, altered neurotransmitter levels, and dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis (including glucocorticoid receptors) are linked to ROS and inflammation. These mechanisms (with respect to impacts of DSP) are explored in Supplementary Discussion at the end of this chapter.

**Environmental perception and context behavior:** Animals need to adjust behaviors according to external conditions. Neuronal damage and general cognitive dysfunction can trigger a mismatched behavioral response to given environmental cues or context [78,79]. Impaired integration of sensory stimuli or loss of appropriate efferent stimulation may be involved.

Animals face a natural conflict between the tendency to explore and avoid novel environments [80]. Rodents will avoid environments perceived as risky (e.g. a brightly lit open area) [81]; however, a shift to dim illumination in the same apparatus can alter the response behavior in favor of exploration [48]. Neuroinflammatory damage prevented mice from eliciting normal responses to light/dark stimuli [78]. Neuroinflammation and other neurodegenerative conditions are commonly associated with aging [4,82]. Therefore, behavioral regulation in response to changing environmental contexts (such as illumination) may be impaired in old mice.

After testing in bright light, we repeated the circle run test and step-down test in dim illumination (Tables 2&3; Figs. 2&3; no genotype effect). In both tests, aging untreated mice showed virtually identical behaviors regardless of lighting. Conversely, behavioral
responses of supplemented mice were significantly different in dim light compared to bright illumination (Figs. 2&3). These results suggest that supplemented mice had better contextual discrimination implying stronger cognitive function. Indeed, our DSP was already shown to improve cognition of aging mice [10,12].

Impaired light/dark discrimination was also associated with poor locomotor ability [82]. Our DSP was shown to upregulate locomotion in mice [11]. Taken together, it appears that our treatment has a general impact on brain sensorimotor neurocircuitry.

**Somatosensory, visual and olfactory function:** Crude evaluation of sensory modalities in ~2 year old Nr mice revealed that our animals did not generally suffer from loss of vision, nociception (pain), proprioception (body position) (Table 4), or loss of olfaction (data not shown). Others also found that simple reflective tasks showed little change in rodents as a function of age [1]. Naturally, these tests were too crude to resolve potential impacts of treatment. However, when visual acuity, olfactory sensitivity and motor balance were examined in carefully refined tests, beneficial impacts of DSP treatment were in fact resolved in all of the above.

**Motor coordination:** Motor balance was tested on the rotarod apparatus [49] in 1–2 year old mice. Tg mice had severely compromised motor coordination compared to Nr (Fig. 4A) consistent with previous reports [13]. Initial balance performance of Nr mice on the rotarod was not affected by DSP treatment (Fig. 4A); however, contrary to untreated mice, supplemented animals showed significant improvement after three consecutive trials (Fig. 4B).

Aging impacts motor behavior [1–3,11] and coordinated control of motor function (i.e. balance) [1,3]. Declines in locomotor abilities are closely linked to oxidative stress [3,11] and may reflect reduced mitochondrial activity [11]. Motor coordination was negatively correlated with increasing levels of protein carbonyls in motor control regions of the brain including the cerebellum [2,3]. We showed that DSP treatment significantly reduced protein carbonyls in brains of aging mice [11]. This was associated with improved locomotor behavior in Nr and Tg [11]. Brain mitochondrial and neurotransmitter function
was also augmented [11,12]. Present results indicate that impacts of DSP on brain physiology also extend to better motor coordination (Fig. 4B).

Cerebellum, Purkinje cells and motor control in aging: While the cerebellum does not initiate movement, it is critically responsible for control, coordination and correction of body movement including sensory analysis of consequences of movement [83]. Animals with cerebellar defects show impaired motor coordination [84–87]. The cerebellum is also highlighted in motor and spatial learning [2,88–91].

Purkinje cells (PC) are the dominant neurons involved in cerebellar information processing [52]. They are one of the largest neurons in the mammalian brain with very intricate dendritic projections and great numbers of dendritic spines. PC provide the sole output pathway of the cerebellar cortex [88,92] and participate in motor coordination [87,92] and motor learning [2,51,88,91,92]. Loss of PC results in imbalance of coordinated movement [85–87,93], impaired cerebellum-dependent learning, sensory processing and other cerebellum-associated behaviors [51,87].

PC are highly vulnerable neurons [51] and exhibit substantial decline with normal aging in humans and rodents [51,52,94–96]. Earlier senescence of the cerebellum compared to other brain regions is primarily attributed to loss or dysfunction of PC [97].

Oxidative stress promoted loss of PC [98,99] which was partially rescued by antioxidant rich diets [17] and melatonin [98]. However, others found no effects [99]. This suggests that simple antioxidant interventions have inconclusive or weak impacts. Heightened sensitivity of these neurons to oxidative stress can explain the rapid age-associated declines.

Our DSP contains ingredients that promote cell integrity, mitochondrial activity, and protection of cellular constituents from oxidative damage [23]. This is expected to support better cell survival. Levels of radiation-induced apoptosis were in fact significantly lowered by the DSP [100]. Brian mass of aging mice was greater if supplemented [12] which may indirectly reflect higher cell counts. Indeed, age-related loss of PC in aging humans was also correlated with decreased cerebellar volume [94]. However, whether DSP treatment actually affected neuronal populations remained to be
determined. Presently we found that numbers of PC were 20% higher in cerebella of ~1.5 year old supplemented Nr mice compared to age-matched controls (Figs. 5D and 6A; Tg were not examined). This is the first direct evidence indicating that DSP treatment can offset age-related neuronal loss. It is very likely that neuroprotection may extend to other regions of the brain.

Counts of PC were performed in lobules II and III from interhemispheric sagittal slices of cerebella (Fig. 5A). PC form a single cell layer between the granule cells and the molecular layer (Fig. 5B). Studies show that total cerebellar PC numbers can be accurately approximated by dividing counts of PC by the length of the Purkinje cell line from representative sections [51,52]. Higher count of PC in supplemented mice is consistent with better motor coordination (Fig. 4B).

Physical exercise was shown to offset loss of PC in rats [96]. DSP-treated mice had higher levels of spontaneous locomotion [11] and wheel running [Ch. 4]. This may additionally counteract PC degeneration.

**Role of the cerebellum in spatial learning:** The involvement of the cerebellum in spatial learning [2,88–91] suggests that the previously reported improved performance of old DSP-treated mice on the Morris water maze (MWM) [12] may be linked to higher populations of Purkinje neurons. It is generally established that learning of spatial tasks (such as the MWM) is dependent on intact hippocampal function [101–103]. However, cerebellar neurons appear to be equally critical. When cerebellar PC were selectively destroyed, mice showed learning deficits on the MWM despite intact hippocampal morphology [103]. A recent study established an anatomic and functional connectivity between the hippocampus and the cerebellum in relation to learning and navigation [104]. It appears that rodents with cerebellar neurodegeneration may remember the location of the escape platform, but are unable to spatially orient themselves and navigate towards it.

The cerebellum ages at a faster rate compared to the hippocampus [97]. This suggests that improved spatial learning of supplemented mice may be primarily attributed to protection of cerebellar neurons. However, supplemented mice also performed better on a test of object recognition [Ch. 6], which does not involve spatial navigation and is mostly
attributed to hippocampal function [105,106]. This suggests that age-related hippocampal
dysfunction can also be attenuated by our DSP [see: Ch. 6].

Gender effects on rates of age-related PC decline were reported [95]; however, we did
not detect a sex difference. It is possible that age-related patterns diverge at ages older
than 1.5 years.

**Cerebellar neurocircuitry links motor coordination with emotionality:** The view
that the cerebellum is purely a center for motor control has been long abandoned [107].
On top of the aforementioned role of the cerebellum in motor coordination, spatial
processing and motor learning [2,51,83–93,97], this brain region is also implicated in
perception of time [108], autonomic functions [109,110] and emotional behavior
[44,79,107,111]. Several reports documented co-morbidity of emotional and balance
disorders [43,44]. In recent years, the role of the cerebellum in modulation of anxiety was
highlighted [44,79,107,111,112] prompted largely by uncovering of added neuronal
connections between the cerebellum and other regions of the brain involved in emotional
control [43,44,79]. However, it was already reported decades ago that abnormal
cerebellar development or degeneration of cerebellar neurons produced nervous or
anxious phenotypes in rodents [113,114]. To a large degree, this was attributed to loss or
dysfunction of PC [113,114]. Attenuation of age-related PC loss in supplemented mice
suggests that neuronal circuits involved in modulation of anxiety-related behaviors
should also be intact. This is presently supported by behavioral testing and may constitute
an additional anxiolytic mechanism of the DSP.

**Cerebellar morphology:** Average thickness of the molecular layer (ML) and granule
cell layer\textsuperscript{17} (GL) was measured. Granule cells are the smallest and most numerous
neurons in the mammalian brain [115], but relatively little is known about their specific
roles. Loss of cerebellar granule cells induced by irradiation resulted in impaired spatial
learning [89]. Genetic mutations affecting granule cell development are associated with
poor cognitive function and motor coordination [85,116]. However, it is challenging to
isolate effects of granule cell depletion as this is usually coupled with loss of neighboring

\textsuperscript{17}The term ‘granule cell layer’ is used interchangeably with ‘granular layer’ in scientific literature.
neurons (e.g. Purkinje cells) [85,89,116], which confounds the results. A recently proposed hypothesis argues that cerebellar granule cells compute stimuli from adjacent neurons to generate operational time-windows which set a temporal framework for integrating sensory information with motor domains [explained in: 115]. This may also set the tone for LTP, affecting learning capacities.

Thickness (or volume) of the GL reflects the number of granule cells [85,96]. We found that supplementation resulted in significantly increased thickness of GL by 18% (Fig. 6C). This was correlated with improved motor balance (Fig. 4) and may play a role in enhanced spatial learning of old supplemented mice [12].

Granule cells send parallel fibers extending to the ML where they interact with the dendritic arbors of PC [117]. The ML also contains interneurons (stellate and basket cells) that provide GABAergic input to PC [117]. The ML interneurons play a role in sensory information processing and motor coordination [117,118]. Aging rats lost 60% of the parallel fiber length and up to 80% of PC synapses [119]. This was reflected by a 30% reduction of ML thickness [119]. Presently, we found that thickness of the ML was increased by 16% in aging DSP treated mice compared to untreated controls (Fig. 6B), suggesting that supplementation may prevent loss of ML interneurons and PC synapses. Improved motor coordination of supplemented mice (Fig. 4) is consistent with greater ML thickness.

**Risks associated with balance dysfunction in the elderly:** Injuries and deaths resulting from falls among the elderly are attributed to deterioration of balance function and motor coordination [24]. Falls are among the top common causes of deaths and disability in persons aged over 65y in the US [24]. Our DSP improved motor balance in aging mice, and human applications appear possible. As onset of cerebellar aging may be already in progress by mid-adulthood [97], it follows that greatest benefits of treatment will be attained if supplementation begins earlier in life. However, because cerebellar function continues to further decline in advanced age, sizeable benefits may still be possible with late intervention. Assuming that effects of treatment translate to humans,
our DSP may reduce the health risks associated with poor motor coordination and falling in the elderly.

**Visual acuity in aging:** Our mice do not display profound vision loss in old age. However, we suspected that visual acuity will diminish as vision decline is common to normal aging [5]. Tests for assessing visual acuity in rodents generally rely on prior conditioning to discriminate between visual stimuli [120]. Because cognitive function declines with age, these protocols are naturally difficult to adopt for senescent animals as they may not fully learn the cues or quickly forget them. The principle behind tests of visual acuity involves challenging mice with increasingly more difficult visual cues and determining when correct responses are extinguished [120]. Based on this, we developed a novel test which uses the animal’s natural tendency to reach for perceived objects or surfaces to escape when suspended by the tail (see: Methods for full description). A crude version of this protocol was previously attempted [47].

We found that ~2 year old Nr mice had largely intact vision as all animals successfully responded to repeated presentation of a large visual cue on contrasting background, irrespective of treatment (Table 5). However, untreated mice showed a progressive loss of reaching responses as increasingly smaller visual cues were presented. In comparison, responses of supplemented mice extinguished at slower rates. Significant differences between supplemented and untreated mice were resolved on a moderately difficult visual challenge (Table 5). These findings suggest that our DSP may offset visual acuity declines in old age. In humans, sharpest visual acuity is associated with the macular region of the retina [121] which is particularly vulnerable to aging.

**Age-related macular degeneration:** We performed histological examination of eyes harvested from 2 year old mice. Markers of retinal morphology were found to be consistent with those of age-related macular degeneration (AMD) in human patients. Hence, possible therapeutic implications for humans can be inferred. The use of mice in modeling AMD came under question as mice do not have a macula [122,123]. However, patterns of retinal degeneration in mice correspond to those in human disease, and thus useful inferences can be drawn [122–127].
AMD may involve a variety of mechanisms [124], but it is known that inflammatory processes and oxidative damage play a large role in the development of this pathology [128–132]. Furthermore, macular disorders largely implicate gradual lifetime exposure to oxidative stress as opposed to acute episodes [128]. Superoxide dismutase knockdown mice showed early onset of AMD symptoms [127,133]. Studies investigating the use of antioxidants to prevent AMD had variable, yet promising success [128,134]. However, a recent review of clinical and experimental data concluded that singly administered antioxidants (such as vitamin E and β-carotene) did not improve AMD prognosis [135]. Recently, mitochondrial dysfunction was additionally implicated in development of AMD [131]. Considering that this pathology involves dysregulation of multiple cellular systems, greater therapeutic benefits should be possible through complex multi-targeted interventions.

**Geographic retinal atrophy:** Our DSP was designed to target general mechanisms associated with aging which include: oxidative stress, inflammation and mitochondrial function [23]. Indeed, strong impact of our DSP on these parameters was experimentally confirmed [11–13]. Presently, we found that DSP affected reliable histological markers associated with AMD. This was likely attributed attenuated oxidative and inflammatory processes and boosted mitochondrial function.

Thicknesses of the retinal outer nuclear layer (ONL) and outer segment (OS) were increased by 26% and 29%, respectively in supplemented mice, compared to controls (Fig. 8). These differences are visually apparent (Figs. 7B–E). Irregular organization and degeneration of the ONL and OS are closely associated with AMD in humans [136]. In mouse models for AMD, geographic atrophy of the ONL and OS are regarded as chief biomarkers of retinal degeneration consistent with human AMD [122,125–127,137]. The inner nuclear layer, however, remains intact in AMD [137]. Thinning of the ONL occurs as a result of cell depletion [137]. The ONL contains photoreceptor nuclei [138]; therefore, cell loss in the ONL reflects a reduction of the attached photoreceptors (rods and cones) [136] and diminished visual acuity [138]. Likewise, the OS reflect rod and cone abundance [139]. In this fashion, improved visual acuity in old supplemented mice
is consistent with reduced retinal degeneration. Given that similar geographic atrophy is observed in AMD, it is arguable that our DSP may offset this pathology in humans.

**Intraretinal cystoid spaces:** Contrary to previous works (see above), a recent study found that decreased visual acuity was actually associated with increased ONL volume [138]. Authors showed that AMD may involve formation of cystoid spaces throughout the ONL. This can lead to thickening of the ONL despite an actual loss of retinal cells. In addition, fluid exudation into the retina can diffuse cells resulting in apparent thickening of retinal layers. Therefore, authors caution against indiscriminant use of ONL thickness as a marker for AMD. We compared several randomly selected segments of the ONL from both treatment groups and found no significant differences in cell densities. This validates the present analysis.

**Dietary supplement and retinal degeneration:** Since earlier works [11–13], our DSP was modified to include lutein. In addition, dosages of some existing ingredients were also adjusted [see: Ch. 4]. Lutein is one of the predominant macular carotenoids which provide macular pigmentation to protect the retina from light-induced damage [140]. Higher carotenoid levels can be obtained from diet [140]. Since mice lack a macula, it seems that benefits of lutein supplementation may not extend to mice. However, there is indication that lutein (along with other carotenoids) is also present in the retina in small amounts [140]. Also, lutein has antioxidant capacities and was found to exert neuroprotective effects [132]. Therefore, mice may still benefit from lutein supplementation, although greater impacts are expected in humans [121].

**Olfactory sensitivity:** Effect of DSP treatment on olfactory sensitivity was examined in aging Nr animals. Test protocol was adopted from procedures specifically developed for assessing olfaction in mice [50]. Highly diluted concentrations of peanut butter did not induce elevated exploratory behavior in either treatment, presumably because the odor was too weak to be detected. However, as concentration was slowly elevated, supplemented mice increased exploration of the scented object in a dose response fashion (Fig. 9). Untreated mice did not show a change in exploratory duration on either of the dilute concentrations suggesting that olfactory sensitivity was lost (Fig. 9). On a
preliminary test with undiluted peanut better, both untreated and supplemented mice showed equally high exploration (data not shown). This indicates that untreated mice did not entirely lose their sense of smell. More importantly, however, this shows that results of the olfactory acuity test did not reflect differences in exploratory tendencies.

**Laminar morphology in the aging olfactory bulb:** Morphological changes in olfactory bulbs of aging rodents are somewhat debatable with respect to laminar features. Volume (or cross-sectional thickness) of GLM and EPL (see: Fig. 10A) are often measured [141–143]. Some report no significant changes in volumes with age [142], while other show that thickness of these layers (as well as the entire bulb volume) increases between 6 and 24 months in C57BL/6 mice [141]. Increases were equally proportional in each layer [141]. An earlier study by Hinds & McNelly [143] may explain this discrepancy. Authors found that volumes of all laminar layers in rat olfactory bulbs significantly increased from 3 to 24 months of age, followed by a sharp decline thereafter. At oldest ages (~30 months) layer volumes were identical to those in 3–12 month old rats. In fact, the more recent study claiming no effects of age on laminar volumes reported similar patterns (i.e. age-related elevation followed by age-related decline) [142]. However, authors used a sample size of only three mice, which may explain lack of significant resolution [142], compared to a sample size of 12 animals used by Hinds and McNelly [143].

Collectively, it appears that laminar volumes increase with age (if extremely old animals are excluded) [141,143]. In our mice (ages: 14–17 months), DSP treatment resulted in reduced volumes of the GLM and EPL (Figs. 11B and C), suggesting that treatment offsets age-related morphology. This was correlated with better olfactory sensitivity of aging mice in behavioral tests (Fig. 9).

Neuronal and synaptic density in the GLM was shown to decline with age in mice [141,142]. In one study, this was correlated with increased GLM thickness and reduced immunelabeling of tyrosine hydroxylase (TH) [141]. Neurochemical labeling clearly showed that expression of TH was restricted to the GLM [144]. TH is the rate-limiting enzyme in the synthesis of L-DOPA [145], the precursor for dopamine. Thus, GLM
enlargement may be associated with loss of dopaminergic function. Aging mice treated with DSP had reduced GLM thickness (Fig. 11B) which may indirectly reflect positive impacts on dopamine neuron activity. To the contrary, one study claims that a number of mouse and rat strains do not exhibit age-related loss of dopamine phenotype expression in the olfactory bulb [146]. Hence it is unclear whether strong parallels can be drawn between the olfactory bulb and other dopaminergic systems in the CNS.

Mitral cells: Mitral cells are the primary output neurons in the olfactory bulb that process olfactory sensory input prior to activating higher-order processing [7,147]. Age-related (or otherwise caused) loss of odor perception may occur due to complications afferent to mitral cells, but mitral cell dysfunction is ultimately implicated [7,148]. Loss of mitral cells with age is documented in rodents [141,143] and sizable declines are seen in humans [148,149]. Our DSP-treated mice showed elevated counts of mitral cells by 29% (Fig. 11A), suggesting that treatment protected these neurons from age-related atrophy. Due to difficulty in morphological discrimination of mitral cells from slides stained with H&E (see: Appendix; also see: Figs. 10B and C) these preliminary results should be interpreted with caution. Regardless, the findings seem clear. Mitral cells are particularly susceptible to oxidative and nitrative stress [150,151]. Amount of 3-NT (a marker for protein nitration) was significantly elevated in olfactory bulbs of old mice [150,151]. 3-NT was evident in the mitral cell layer, but was also delocalized to the EPL and GLM in advanced ages [151]. We showed that DSP treatment significantly lowered 3-NT levels in brain homogenates of aging mice [13] and protein carbonyls were also reduced [11]. This is consistent with potential protection of the mitral cell population.

Olfaction and neurodegenerative disease in humans: Humans, like other animals, display age-related declines in olfactory function [6–8]. Losing the sense of smell per se, does not pose a major threat for humans, although quality of life may be impacted [7]. One study found that 45% of elderly persons were unable to detect warning odor levels of natural gas [8]. However, greater concerns emerged when loss of olfaction was found to be strongly correlated with the risk of developing severe neurodegenerative conditions.
Interest in olfactory research in humans gained momentum in the 1980s with the discovery of a link between neuronal changes in olfactory bulbs and Alzheimer’s (AD) and Parkinson’s disease (PD) [152,153]. A decade later it was shown that AD patients show deterioration of mitral cells in olfactory bulbs, and that substantial mitral cell loss was apparent even before AD was fully diagnosed [25]. Combined with previous reports of severe olfactory deficits in AD and PD [154,155], authors suggested that olfactory dysfunction may be an early manifestation of neurodegenerative disease [25]. Neuronal loss and altered olfactory bulb morphology was later shown in young AD patients [29], suggesting that loss of olfaction and olfactory neurons is not simply an age-related condition that parallels cognitive impairment in old AD patients. Recent studies confirm that olfactory deficits are associated with cognitive impairment, and loss of olfaction specifically precedes onset of AD [26–28] and PD symptoms [30,31] and dementia [33]. In fact some have even proposed that pathogenesis in AD and PD may be catalyzed by agents that enter the brain via olfactory pathways [156].

The accuracy of olfactory tests in early detection of neural pathology suggests that individuals with intact sense of smell are at lower risk of developing neurodegenerative conditions in the near future. Higher counts of mitral cells in the olfactory bulb also reflect higher neuronal populations in adjacent brain regions. Given that aging supplemented mice showed better olfactory sensitivity and higher number of mitral cells in the olfactory bulb, suggests that our DSP may be offsetting neurodegeneration throughout the brain. Comparable impacts in humans would be consistent with reduced risk of developing AD, PD and dementia. Impacts of DSP on neuronal populations in the hippocampus and striatum remain to be investigated\(^{18}\). Regardless, the present findings provide considerable evidence that DSP treatment may considerably delay onset of debilitating neurodegenerative conditions.

**Summary:** Effects of treatment with a complex dietary supplement designed to ameliorate mechanisms of aging were tested in mice. We found that treatment was associated with reduced anxiety-like behaviors, augmented discrimination of

\(^{18}\) Hippocampal and striatal neurons were examined at a later date [see: Ch. 6].
environmental context, improved motor balance, and improved visual and olfactory acuity. This was correlated with positive morphological changes and higher neuronal populations in the cerebellum and olfactory bulb. Intact olfaction is strongly indicative of suppressed neuronal degeneration. Retinal atrophy (associated with AMD) was also diminished in supplemented mice. Previous findings that DSP treatment reduced oxidative damage, boosted mitochondrial function [11,12] and alleviated symptoms of inflammation (unpublished data) suggest that neuronal protection and sensory function is likely attributed to diminishing oxidative/inflammatory stress and improved energy balance. In addition, the DSP may also modulate the endocrine stress axis further contributing to the overall phenotype and heightening stress resistance (see: Supplementary Discussion). The extent of functional benefits attained by our DSP here and before [11–13] strongly suggests that aging animals retain the capacity to support most youthful phenotypes and that powerful impacts can be achieved through multi-ingredient dietary supplementation.

3.5 ACKNOWLEDGMENTS

Authors would like to recognize Ted A. Aristilde for providing technical histology support and Dr. Henry Szechman for providing the image tracking system for behavioral studies. Research funding supported by a grant to C.D. Rollo (NSERC).
3.6 FIGURES and TABLES

**Table 1.** Open field: Effects of age, genotype and treatment on distance traveled by zone, and mean running velocity (n=56). Strong effects of age and genotype were resolved for all variables. Treatment affected distance traveled in central zone only. Slopes of age-related regressions were compared and found to be independent of either genotype, treatment or genotype*treatment (bottom panel)

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<th>Peripheral zone: Distance traveled (ANCOVA)</th>
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Table 2. Step-down test: Effects of variables on latency to step down. Strong effects of treatment and illumination were found. Age and genotype were marginally resolved, which allowed pooling Nr and Tg across ages. Total: n=141 mice

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</table>

Table 3. Circle run test: Effects of variables on latency to (a) emerge from cup and (b) escape to shaded overhang tested with ANCOVA. Significant effects are bolded. Combined effects of illumination*treatment were strongest for both latencies. Weak effects of age and genotype allowed for pooling of Nr and Tg data across all ages

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Latency to emerge from cup (ANCOVA)</th>
<th>Latency to escape to shade (ANCOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>p=0.0620</td>
<td>p=0.939</td>
</tr>
<tr>
<td>Illumination</td>
<td><strong>p=0.001</strong></td>
<td>p=0.096</td>
</tr>
<tr>
<td>Genotype</td>
<td>p=0.167</td>
<td>p=0.765</td>
</tr>
<tr>
<td>Treatment</td>
<td><strong>p=0.005</strong></td>
<td>p=0.532</td>
</tr>
<tr>
<td>Illumination*Genotype</td>
<td>p=0.221</td>
<td>p=0.055</td>
</tr>
<tr>
<td>Illumination*Treatment</td>
<td><strong>p&lt;0.001</strong></td>
<td><strong>p&lt;0.001</strong></td>
</tr>
<tr>
<td>Genotype*Treatment</td>
<td>p=0.837</td>
<td>p=0.080</td>
</tr>
</tbody>
</table>
Table 4. Number of supplemented and untreated Nr mice showing positive responses in simple somatosensory tests. DSP treatment did not improve somatosensory scores as nearly all untreated mice already exhibited positive responses.

<table>
<thead>
<tr>
<th>Test</th>
<th>Mice showing positive responses</th>
<th>Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Supplemented</td>
</tr>
<tr>
<td>Landing response</td>
<td>27 of 29</td>
<td>27 of 27</td>
</tr>
<tr>
<td>Visual placing</td>
<td>34 of 37</td>
<td>28 of 29</td>
</tr>
<tr>
<td>Negative geotaxis</td>
<td>34 of 40</td>
<td>39 of 42</td>
</tr>
<tr>
<td>Pinch reflex</td>
<td>29 of 29</td>
<td>27 of 27</td>
</tr>
</tbody>
</table>

Table 5. Results of the visual acuity test in supplemented and untreated Nr mice (n=22 per group; age: 1.5–2 years). The proportion of mice exhibiting reaching responses decreased with increasing difficulty. A significant difference was resolved on a moderately difficult challenge (#4) indicating that supplemented mice had better visual acuity. All mice showed a reaching response on the easiest task (#1) and all reaching responses were completely extinguished on the most challenging task (#6). Effects of sex were not resolved.

<table>
<thead>
<tr>
<th>Visual challenge</th>
<th>Reaching responses</th>
<th>Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated (%)</td>
<td>Supp (%)</td>
</tr>
<tr>
<td>#1: 5mm wire, white background, room light</td>
<td>22 (100)</td>
<td>22 (100)</td>
</tr>
<tr>
<td>#2: 5mm wire, black background, room light</td>
<td>20 (91)</td>
<td>21 (95)</td>
</tr>
<tr>
<td>#3: 1mm wire, black background, room light</td>
<td>14 (63)</td>
<td>18 (82)</td>
</tr>
<tr>
<td>#4: 0.5mm wire, black background, room light</td>
<td>5 (23)</td>
<td>14 (63)</td>
</tr>
<tr>
<td>#5: 0.5mm wire, black background, dim light</td>
<td>1 (5)</td>
<td>4 (18)</td>
</tr>
<tr>
<td>#6: 0.5mm wire, black background, near-dark</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

A total of 22 mice were tested in each treatment group.
Figure 1. Open field: Average distance traveled in central and peripheral zones by Nr untreated and supplemented (n=14, n=10), and Tg untreated and supplemented (n=17, n=15) mice in a 5 minute period. Supplemented Nr covered 113% (ANCOVA; SNK \( p=0.003 \)) more distance in the central zone compared to untreated Nr controls. No treatment effects in Tg mice were seen. Distance traveled in peripheral compared to central zone was greater for all groups (\( p<0.0001 \)). Effects of treatment on travel distance in the peripheral zone were not resolved. Combined, Tg covered a ~40% shorted distance in the peripheral zone compared to Nr mice (\( p=0.0005 \))
Figure 2. Step-down test: Latency to step down was pooled across genotypes (see: Table 2). Untreated mice showed no significant differences in latency between bright (n=67) and dark (n=39) conditions. Supplemented mice were (38%) quicker to step down in darkness (n=21) compared to bright illumination (n=73) (ANOVA, Duncan’s test: p=0.05). Supplemented animals were quicker to step down than untreated controls only in dark conditions (ANOVA, Duncan’s test: p=0.038).
Figure 3. Circle run test: In bright illumination both the latency to emerge from cup and the latency to reach the shaded overhang was significantly elevated in supplemented mice by 34% (*$p<0.0001$) and 17% (*$p=0.048$), respectively. Conversely, in dark conditions, latency to escape to shade was significantly lower in supplemented mice by 18% (*$p=0.019$) compared to untreated controls. Going from bright to dark illumination the latency to emerge from cup and to escape to shade was significantly lowered only in supplemented mice by 25% (*$p<0.0001$) and 21% (*$p=0.006$), respectively. Responses of untreated mice were virtually identical in either illumination.
Figure 4. Rotarod motor balance test: (A) Mean latency to fall (3 trial average) for untreated and supplemented Nr (n=12, n=9) and untreated and supplemented Tg mice (n=17, n=7). Effects of age (p=0.625), body weight (p=0.469) and treatment (p=0.759) were not resolved. Overall Tg mice were over four times quicker to fall compared to Nr (ANOVA; SNK effect of genotype: \( p=0.002 \)). (B) The difference in latency to fall between the first and last trial was calculated by subtracting the latency on the last trial from the latency on the first attempt. Nr and Tg mice were pooled as effects of genotype were not resolved (ANOVA: p=0.390). Untreated mice showed no improvement on repeated attempts while supplemented mice had a ~40 second improvement by the last trial (*t-test: \( p=0.044 \))
Figure 5. (A) Macrostructure of the mouse cerebellum: Interhemispheric sagittal cerebellar section showing lobules I–V (H&E, ×40 magnification). Purkinje cells were counted in lobules II and III. (B) Microstructure of the cerebellum: Showing molecular layer, ML; white matter, W; granule cell layer, GL; and Purkinje cell layer, PC. Arrows point to individual Purkinje cells which are organized in a single layer on the border of ML and GL (H&E, ×100 magnification). (C) Image processed using ImageJ software. Dark area shows the section of ML used to calculate mean ML thickness. Portion of the GL underlying the dark region was used to calculate mean GL thickness. (D) Representative slides from matching cerebellar areas of supplemented and untreated mice (Nissl stain, ×100 magnification). Notice the relative scarcity of Purkinje cells in untreated tissue compared to supplemented. All tissue sectioned at 5 microns.
Figure 6. (A) Number of Purkinje cells per mm of cell line length in cerebella of Nr mice. Supplemented mice had 20% more Purkinje cells compared to controls (t-test; \( *p<0.05 \)). (B) Average thickness of ML and (C) GL in cerebellar lobules II and III. Thickness of ML and GL was greater in supplemented mice by 16% and 18%, respectively, compared to untreated controls (t-test; \( *p<0.05, **p<0.01 \)). Effects of sex were not resolved, n=6 mice per group, age: 14–17 months.
Figure 7. (A) Sagittal cross-section of the eye showing retinal organization (×40 magnification). (B–C) Enlarged sections of proximal retina from an untreated and supplemented mouse (×100 magnification) showing the inner nuclear layer, INL; outer nuclear layer, ONL; and outer segments, OS. Notice the visible reduction in ONL thickness from an untreated mouse (B) compared with supplemented (C). (D–E) Images processed with ImageJ software digitally isolating the OS (white) from surrounding layers (black). Average thickness of OS was calculated by digitally computing the ‘white’ area and dividing by the length of the layer. Notice the visibly reduced OS in an untreated (D) compared with a supplemented (E) mouse. ONL and OS were measured along the proximal retinal portion which was set at 1000 µm superior and inferior to position of optic nerve attachment, Opt. Sections were cut at 5 microns and stained with H&E.
Figure 8. Average ONL (A) and OS (B) thickness in proximal retinal regions of supplemented and untreated Nr mice (n=4 per group; age ~2 year). Mean ONL thickness was 32.71±2.44µm and 41.14±2.44µm for untreated and supplemented mice, respectively (26% difference; t-test: *p<0.05). Mean OS thickness was 29% greater in supplemented mice (25.65±1.87µm) than in untreated controls (19.81±1.21µm) (t-test: *p<0.05)
Figure 9. Olfactory sensitivity: Relationship between duration of exploratory behavior and concentration of olfactory cue (peanut butter) for supplemented (n=11) and control (n=11) Nr mice (age: 1.5–2 years). Each point is the mean value of exploratory duration averaged for 11 mice in each treatment group. No significant regression was seen in untreated mice. A strong positive relationship was observed in supplemented mice indicating a dose response mechanism. Linear regressions:
Untreated: $y=142.145+25.101x; r^2=0.069; (p=0.738)$
Supplemented: $y=119.763+245.964x; r^2=0.976; (p=0.012)$
ANCOVA (covariate=concentration) resolved a significant difference in slopes of regression lines between treatments $(p=0.036)$
Figure 10. (A) Cross section of mouse olfactory bulb showing the glomerular layer, GLM; external plexiform layer, EPL; mitral cell layer, MCL; and granule cell layer, GCL (×40 magnification). (B) Close up of the MCL (×100 magnification) showing several pyramidal-shaped mitral cells. Granule cells occur in the MCL but appear smaller and darker than mitral cells. Arrow heads point to possible mitral cells, but accurate classification is difficult as cells show a range of sizes, variable spatial orientation, overlapping, and may occur outside the MCL. For maximal consistency, careful discrimination criteria were applied: (C) Image processed with ImageJ software to highlight cellular structure. Only cells greater than five pixels across were counted (cell 3 does meet size criteria). Only cells with clearly defined nuclear envelope and nucleolus were counted (large arrows). Cells 1 and 2 do not clearly show the nucleus (not counted). Cell 4 is outside the MCL, hence not counted. Tissue stained with H&E, sections: 5 microns
Figure 11. (A) Number of mitral cells meeting count criteria [see: Fig. 10 caption] in a 1mm representative section of mitral cell layer in olfactory bulb. Supplemented mice showed a 29% increase in cell number compared to controls (t-test; *p=0.030). (B) Mean GML and (C) EPL thickness in the olfactory bulb. Supplemented mice had a 25% (*p=0.026) and 28% (**p<0.001) reduced GML and EPL thickness, respectively, compared to controls. Mice aged 14–17 months (untreated n=5; supplemented n=6)

3.7 SUPPLEMENTARY DISCUSSION

This discussion picks up from main text exploring mechanisms of anxiety with respect to anxiolytic effects of the DSP and possible targets.

Role of neuropeptide Y in anxiety: Neuropeptide Y (NPY) is an abundant neurotransmitter throughout the brain [11] and shows prominent age-related declines [11,157,158]. Infusion of NPY is associated with reduced anxiety while NPY receptor antagonists produce opposite effects [159]. Indeed, NPY is considered to be an endogenous anxiolytic peptide [160], and NPY knockout mice exhibit phenotypes of
elevated anxiety [161]. Oxidative stress depletes NPY [162] which may partially explain age-related declines. Oxidative processes are an important target of our DSP [23] and the DSP was found to offset NPY loss in brains of aging mice in association with diminished oxidative damage [11]. This suggests that reduced anxiety-like behaviors in aging supplemented animals (Figs. 1–3) may in part trace to NPY levels.

**Anxiety and the GABAergic system:** Anxiety-related disorders are most often associated with dysfunction of the gamma-amino-butyric acid (GABA) system [37,38,56,163–165]. While our mice do not suffer from abnormal anxiety [47], the present findings prompted us to explore the possible involvement of a GABAergic action of the DSP.

GABA is the key inhibitory neurotransmitter in the CNS [38,163]. Decreased GABAergic stimulation is associated with elevated anxiety-like behaviors, while augmented GABAergic tone diminishes anxiety [38,163,164]. Altered GABA neurotransmission may reflect rates of production or release, but can also involve modulation of GABA receptors [38,165,166] or reuptake rates [164].

Although anxiolytic effects of the DSP are consistent with elevated GABAergic processes evidence addressed in the following sections argues that upregulation of GABA neurotransmission is not the underlying anxiolytic mechanism associated with the DSP.

We must emphasize that region and neural circuit specificity of GABAergic synaptic transmission in the CNS is imperative to the ultimate behavioral and physiological outcome. Isolated brain regions may not always follow the general trends described.

**The GABAergic system in learning and memory:** Elevated activity of the GABAergic network is linked to prominent reduction of cognitive function, memory, spatial learning and general motor activity [61,163,167–172]. Pharmacological GABA receptor blockers and other negative modulators of GABA enhance memory and learning [61,170,171]. Treatment of anxiety disorders by anxiolytic drugs that target GABA systems is often accompanied by cognitive impairment [166,173]. Brain infusion with GABA or GABA receptor agonists results in learning and memory deficits [163,167]. Neurochemically, this effect can be attributed to reduction of long-term potentiation.
(LTP) through stimulation of GABAergic neuroinhibition [166,174,175]. LPT promotes neuronal synaptic plasticity [176,177] which is thought to be the underlying mechanisms for learning and memory formation [176,178–180].

Three studies on memory and learning (8-arm maze, Morris water maze and object recognition) showed that our DSP significantly improved cognitive function of aging Nr and Tg mice [10,12; Ch. 6]. Likewise, DSP boosted physical activity and locomotor behavior in older animals [11; Ch. 4,5]. An 11–28% extension in lifespan was also achieved [181].

Given the prominent cognitive and physical improvement, it is highly improbable that supplementation augmented GABA neurotransmission throughout the CNS. Indeed, there is no pharmacological evidence that any of the ingredients in our DSP have potent anxiolytic action that can be traced to specific modulation of the GABAergic system. In fact, the collective impacts of DSP treatment on aging [10–13; Ch. 4–6] appear to be more consistent with elevated dopaminergic function which is sensitive to aging and central to cognitive and physical performance [4,182,183]. Preliminary in situ hybridization results suggest that age-related loss of dopaminergic function in the striatum may be reversed by supplementation. At common synaptic junctures, these neurotransmitters appear to be antagonistic. Dopamine is known to inhibit GABA release [184] while GABAergic processes in turn attenuate dopamine [185–188] (although exceptions occur [185,188]). Finally, increased synaptic availability of dopamine is actually associated with elevated anxiety [182].

Collectively, the evidence presented above effectively dismisses the presumption that DSP had a direct impact on GABA throughout the brain. We previously showed that DSP is capable of modulating neurotransmitter levels in brains of old mice; however, this was mainly attributed to offsetting age-related losses as opposed to amplifying normal levels [11]. In addition, there is no evidence to suggest that rescue of neurotransmitter-related dysregulation in aging mice should favor a particular neurotransmitter system.

We propose that instead of being mediated via selective modulation of signal transmission at specific synaptic connections, the anxiolytic effects observed here most
probably reflect impacts on a broader systemic mechanism involved in regulation of emotion and anxiety. In the following sections we describe the role of the HPA axis in regulating emotion. We propose that our DSP may affect anxiety-related behaviors in part through modulation of the HPA axis.

**Role of the HPA axis in anxiety and memory and its regulatory control:** Patients suffering from anxiety disorders show increased HPA axis activity [189]. The HPA axis mediates an array of systemic responses to stress (including anxiety related behaviors) through a hormonal cascade [190–194]. Activated by stressors, the paraventricular nucleus (PVN) stimulates release of corticotrophin-releasing hormone (CRH) from the hypothalamus which in turn facilitates adrenocorticotropic (ACTH) secretion from the pituitary [190,194,195]. ACTH induces release of glucocorticoids (GCs) from the adrenal cortex [190,194,195]. In addition to regulating a variety of endocrine, metabolic and immune responses [196], GCs induce anxiety-related behaviors [191,192]. Expectedly, reduced GC stimulation diminishes anxiety [196]. Likewise, hypersecretion of CRH results in hyperactivity of the HPA axis, GC release and elevated anxiety [193].

On top of anxiogenic effects, chronic elevation of GCs impairs spatial learning and memory [64,197–200] through inhibition of LTP [197,201]. Brief exposure to stress can enhance some types of learning [197,199]; however, sustained stimulation of the HPA axis opposes consolidation of memory and impairs learning processes [190].

The PVN is the main control center of the HPA axis [195,202,203]. Systemic stress signals relay directly to this region [195]. Lesions to the PVN precluded ACTH and GC secretion despite stress [203]. Interaction of the PVN with stress responsive regions of the brain is provided by a network of mainly GABA containing neuronal projections [195,202]. Inhibitory input to the PVN from local GABAergic neurons provides sustained suppression of the HPA axis [202]. Stimulation of upstream stress responsive neurons blocks the inhibitory action of local GABAergic projections to the PVN resulting in disinhibition (activation) of the HPA axis [202]. This consolidates the regulatory cascade linking stress, the GABAergic system, HPA axis and anxiety responses.
Autoregulatory feedback loops constitute additional levels of control [202] and the entire system reveals staggering complexity [202,204].

In this framework, systemic stressors exert regulatory GABAergic modulation of anxiety responses through stepwise release of HPA hormones cascading down from the PVN [164,202,205]. When an animal is confined to a distressing environment (e.g. brightly illuminated open arena) the stress signals remove the GABAergic tone in the PVN activating the HPA axis which translates to manifestation of stress-coping and anxiety-like behaviors. A rapid spike in HPA activity is critical for coping with real threats or potential danger. However, anxiety disorders can develop if levels of systemic stressors persist resulting in chronically elevated HPA activity, which also translates to memory and learning impairment.

**Oxidative stress-induced cytokine regulation of the HPA axis:** It is well established that elevated oxidative processes are closely associated with aging. Chronic inflammatory mechanisms are also higher in older animals marked by an increase in circulating cytokines [74,206]. ROS can trigger production and release of inflammatory cytokines [72–74] which were shown to cause activation of the HPA axis [207–211]. Cytokines can regulate the HPA axis at the level of PVN and hypothalamus [210]. Activation of the HPA axis was not possible in animals with pituitary resections [208] or lesions to the PVN [209].

Our DSP includes an array of components with known antioxidant and anti-inflammatory properties [23]. Previous results show that DSP treatment reduced oxidative stress [11,13] and attenuated inflammatory conditions (unpublished). This is expected to downregulate the HPA axis (see above) which is consistent with reduced emotionality presently observed. In fact, dietary modulation of oxidative stress and inflammatory cytokine levels may be a key mechanism responsible for anxiolytic effects of our treatment.

**Oxidative stress in the PVN and the sympathetic nervous system:** Activation of the PVN results in elevated activity of the sympathetic nervous system (SNS) [212]. Similar to the HPA axis, the SNS also mediates a variety of stress responses [204]. Elevated
oxidative stress in the PVN was shown to increase sympathetic tone [213–215]. Increased SNS activity was associated with hypertension [215], heart failure [213], and ventricular hypertrophy [216]. These heart conditions were attenuated by alleviating oxidative stress in the PVN [213]. Our DSP reduced ventricular hypertrophy (Matravadia et al, unpublished), which may reflect SNS attenuation at the PVN level. The SNS and HPA axis are linked by positive feedback, such that activation of one system activates the other, and vise versa [204]. Indeed, blockade of PVN GABA receptors activates both the HPA axis [202] and the SNS [217]. The cardiac anatomy or supplemented mice may indirectly reflect downregulation of the SNS at PVN. This suggest that the HPA axis could be co-downregulated as well.

**Age-related hyporesponsiveness of the HPA axis:** Aging is characterized by endocrinological dysregulations including elevated levels of circulating ACTH and GCs and increased release of CRH in the hypothalamus [194,218]. Physical exercise can normalize levels of HPA axis hormones [219] indicating that attenuation of the HPA axis in aging individuals may be achieved without drastic intervention. A condition commonly observed in aging is hyporesponsiveness of the HPA axis [194]. The system is not only less sensitive to activation by acute stress, but also displays delayed or incomplete recovery (i.e. remains active after stress is withdrawn) [194]. Autoregulatory feedback inhibition is carried out by GCs that deactivate the HPA axis by binding to GC receptors in the hippocampus [194,220,221]. Aging animals show significant losses of GC receptors [218,220] resulting in inability to fully shut off the stress system. Loss of hippocampal GC receptors is thought to result from cumulative chronic exposure to GCs themselves [194]. In this fashion, modulation of systemic stress throughout life, as opposed to advanced ages, appears crucial for preventing hyposensitivity. Our dietary regime involved early life commencement of treatment which appears central to achieving maximal impacts; however, late intervention may still provide relief.

Assuming that DSP treatment attenuated resting levels of HPA axis activity, it should follow that responsiveness of the stress system in old animals is also improved. This implies enhanced responses to acute stress. We showed that supplemented mice had
superior stress resistance to acute whole body ionizing radiation [23]. Levels of radiation-induced apoptosis and chromosomal aberrations were significantly lower in supplemented animals [23,222]. Stress-resistance was mainly attributed to cellular protection against oxidative stress, but it is plausible that responsiveness of the HPA axis may also be implicated.

**Role of HPA axis hyporesponsiveness in pathological conditions:** Hyporeactivity of the HPA axis is also associated with seasonal depression, chronic fatigue, rheumatoid arthritis, multiple sclerosis [review in: 221] and in autoimmune disorders such as lupus [223]. This suggests that benefits of DSP treatment may also extend to these conditions. In fact, we found that supplementation significantly extended longevity and reduced symptoms of autoimmunity in mouse models of lupus-like disease (MRL/lpr and MRL+/+ mice) (unpublished). Presently, it appears that impacts of DSP on regulatory components of the HPA axis may have been influential.

**Emotionality of Tg mice:** Effects of DSP on anxiety behavior of Tg animals were not resolved in the open field test (Fig. 1). Effects of treatment on anxiety assessed by the circle run test and the step-down test were similar in both genotypes (Tables 2 and 3); however, when Tg were evaluated separately from Nr, impacts of DSP disappeared (data not shown). Our earlier data indicates that Tg mice are far less fearful and emotional than normal counterparts (i.e. already exhibit reduced anxiety) [47]. Therefore, additional impacts of DSP are unlikely to further influence anxiety in this strain.

Tg mice have over 100-fold the normal levels of circulating growth hormone (GH) [224], a key effector of the GH axis. Chronically elevated GH alters the HPA axis [225]. As these axes are antagonistic, activity of the HPA axis is expected to be suppressed in Tg. Indeed, Tg mice show compromised stress resistance [23]. The regulatory impacts of HPA axis hormones on anxiety-related behaviors may explain the suppressed emotionality of Tg mice. The HPA axis shows counter-regulatory elevation in transgenic GH mice [226] although functionally, the GH axis regulation is predominant.
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3.9 APPENDIX

**Identification of mitral cells in the olfactory bulb:** We employed strict criteria for counting mitral cells which may otherwise result in arbitrary identification (see: Fig. 10 caption). Identification of mitral cells from H&E slides may be difficult (see: Fig. 2&3 in [227]). Regardless of staining methods, identification is challenging because depending on spatial orientation, mitral cells may appear indistinguishable from granular cells (Fig. 3 in [228]). Even with electron microscope imaging, discrimination of mitral cells is difficult as they show a great range of sizes (see: Fig. 2 in [229]). Tufted cells show close morphological similarity to mitral cells and they may occur the mitral cell layer [230]. Regardless, we believe our methods yielded very reasonable accuracy due to the strict criteria employed.

**Figure A1.** The circle run arena. A 5cm deep cup is fitted in a depression in the center of the arena floor. A shaded overhang encircling the perimeter of the open area provides a potential dark refuge. A mouse is placed in the cup. The latencies to a) emerge from the cup onto the open area and b) subsequently escape into the dark refuge is recorded
CHAPTER 4

Chapter introduction:

Effects of our DSP on major functional systems in aging mice (locomotion, cognition and sensory function) were described in preceding chapters. By this point we presented substantial evidence to argue that complex supplementation can considerably slow rates of functional aging. This chapter expands the growing list of age-related biomarkers to include impacts of the DSP on cellular energy levels, muscle strength and additional functional measures.

Elevated duration of spontaneous locomotion in old supplemented mice was established [Ch. 1]; but intensity of motor function was not assessed. Here we compliment the previous data by confirming impacts of the DSP on physical intensity. Muscle strength in supplemented mice was also augmented. Declining muscle strength is closely associated with aging and strongly predicts all-cause mortality in humans. Sarcopenia (loss of muscle) afflicts a large cohort of the elderly population limiting mobility, independent living and facilitating falls and onset of age-related pathologies. Our DSP emerges as a promising preventative intervention.

Mitochondrial data are revisited with emphasis on complex I and II in aging mice. Interesting patterns in modulation of mitochondrial electron transport chain enzyme activity attributed to the DSP are uncovered. Levels of hydrogen peroxide and lipid peroxidation are evaluated in conjunction with mitochondrial results. Higher body temperature of supplemented mice suggests elevated metabolism, but mitochondrial uncoupling is also considered.

Energy shortfalls contribute to aging. Deficits in cellular energy (ATP) associated with mitochondrial dysfunction are implicated in functional decline and neurodegenerative disease. Maintaining positive energy balance in old mice (by supporting mitochondrial activity) was an important target of our DSP. Mitochondrial results [Ch. 1 and 2] suggested increased energy production, but direct assessment of cellular energy was outstanding.

Here we document a significant decrease in ATP levels in brains of old untreated mice. ATP deficits were associated with rapid deterioration preceding mortality. Remarkably, old supplemented mice expressed no change in ATP levels compared to younger ages. Regulation of ATP synthesis and limitations of using ATP as a marker of cellular energy are discussed. Preventing energy shortfalls is imperative to offsetting functional aging and prolonging lifespan.

Note: Citation number [4] refers to Chapter 1; and citation number [27] refers to Chapter 2
CHAPTER 4

TITLE

Age-Related Declines in Brain ATP and Motor Function are Ameliorated by a Complex Dietary Supplement: Mitochondria, Free Radical Processes and Thermogenesis

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4.1 ABBREVIATIONS

<table>
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<tr>
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<tbody>
<tr>
<td>ADP/ATP</td>
<td>Adenosine diphosphate/adenosine triphosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>ETC</td>
<td>Mitochondrial electron transport chain</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NAD+/NADH</td>
<td>Nicotinamide adenine dinucleotide oxidized/reduced</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
</tbody>
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(All other abbreviations are explained in text)
4.2 INTRODUCTION

Declining motor function is a universal biomarker of aging [1–6]. In addition to shorter duration, older humans exhibit diminished vigor of physical activity [7]. Diminished vigor (or locomotor intensity) is also well characterized in mice [1,8–10]. We previously showed that age-related declines in spontaneous locomotor duration were completely offset by a complex dietary supplement (DSP) designed to target five major processes implicated in mammalian aging [4]. Here we report effects of the DSP on intensity of locomotion in aging mice. Muscle strength, mitochondrial function and cellular ATP are closely implicated and were presently assessed.

Age-related deficits in skeletal musculature pose considerable constraints on intensity of physical activity [11–15]. Cardiovascular capacity appears to be less limiting in comparison [16]. Benefits of physical activity for functional aging and longevity are well established [16–18]; however, declining muscle function in aging persons poses a challenge for sustaining active lifestyles. Skeletal muscle mass steadily declines with age by 1–2% per year after the sixth decade [19]. This decline is reflected in diminished muscle strength [19]. The latter is a strong independent predictor of all-cause mortality in humans [20]. Assessment of grip strength is a standard test of muscle strength in humans [20–23] and rodents [24,25]. In rodents, a wire hang test may also be employed [1,26], incorporating an element of agility [1]. We show that aging mice supplemented with our DSP have greater muscle strength and exhibit elevated intensity of spontaneous locomotor activity. These findings are consistent with previously reported higher duration of non-intensive locomotion [4] and exercise [Ch. 5] in supplemented mice. Human applications would extend to supporting active lifestyles and obtaining the associated benefits for aging and general health.

Age-related neurological changes are often implicated in loss of motor function which may include oxidative damage, mitochondrial dysfunction, neuronal damage, and impaired synaptic transmission [1,3,4,62,63; Ch. 3]. Impacts of our DSP extended to all of the above [see: 4,27,28; Ch. 3,5,6]. Age-related declines in mitochondrial function result in cellular energy shortfalls. Humans may experience a ~25% drop in cellular ATP
Mitochondrial dysfunction and energy shortage are associated with aging [4,27,28,30–32] and debilitating motor and cognitive impairments such as Parkinson’s, Huntington’s, Alzheimer’s and ALS [33]. In addition, the aging brain may have higher energy requirements associated with greater repair and maintenance demands. A depletion of ATP stores in old age may reflect a combination of higher energy needs and insufficient mitochondrial activity. Vital functions such as protein synthesis and Na⁺/K⁺ ATPase are highly energy demanding [34,35], and thus may be most compromised. This can lead to further neurological disorders and neurodegeneration [36–38]. DNA repair systems are also substantially inhibited when ATP is limiting [29] which may exacerbate neuronal death. Rescuing energy balance in the aging brain would extend to preservation of stronger motor and cognitive functionality. Indeed, this is supported by the present findings and previous reports [see: 4,27; Ch. 3,5].

Under normal conditions, ATP levels in the cell are maintained constant and do not reflect ATP synthesis capacity [34]. ATP production is feedback-regulated by rates of utilization such that ATP synthesis is upregulated or suppressed when energy demands rise or decline, respectively [34] (details in: Discussion). However, if mitochondrial function is compromised (or substrates are limiting) energy demands may exceed maximal synthesis rates resulting in detectable depletion of cellular ATP stores. Compensation through upregulated glycolysis is limited in neurons [39,40].

We detected a 30% decrease in brain ATP in old (>2y) mice compared to younger adults, and an even bigger drop when mice reach age-related endpoint (i.e. near death). Alternatively, ATP levels in the brains of our oldest supplemented mice were identical to those of younger adults. We previously reported that activity of mitochondrial complexes III and IV were nearly doubled in supplemented old mice compared to age-matched untreated controls [4,27], suggestive of better energy status. The present results directly support this. Interestingly, activity of complex I was largely unaffected by supplementation; however, complex II activity was significantly upregulated. Complex I and II are the entry points for electrons into the mitochondrial ETC [41–43]. Despite some overlap, their roles in cellular respiration pathways and contributions to energy
production differ [41,44]. Impacts of our DSP appear to specifically modulate complex II activity. The associated implications for cellular respiration, energy production and free radical generation are discussed.

Free radicals and oxidative damage are closely implicated in aging [4,27,28,45,46]. Levels of lipid peroxidation and hydrogen peroxide (H$_2$O$_2$) were assessed here. Elevated H$_2$O$_2$ is expected to correlate with higher oxidative damage. Remarkably, higher H$_2$O$_2$ in brains of supplemented mice was not paralleled by elevated lipid peroxidation. In addition, we previously found that markers of protein oxidation [4], protein nitration [28] and DNA damage [47] were actually reduced in supplemented mice. Hydrogen peroxide participates in many vital cellular pathways [48–50]. It is generated in cells by super oxide dismutases which convert the more harmful superoxide radical to H$_2$O$_2$ [50]. The latter is more stable and less reactive. In addition to mitochondria, cells employ NADPH oxidases to generate oxygen radicals [50]. Hence, higher levels of H$_2$O$_2$ may be unattributed to mitochondrial sources, but simply reflect higher cellular activity.

Lastly, we assessed body temperature of mice. Links between mitochondrial function, temperature and longevity are discussed, and impacts of the DSP on thermoregulation are evaluated with respect to behavioral and molecular data.

4.3 METHODS

**Animals:** All breeding and husbandry protocols adhered to Canada Council on Animal Care guidelines and were previously described [26,51]. Normal (Nr) and growth hormone transgenic (Tg) mice were bred in-house and randomly selected for various studies.

**Dietary supplement:** Preparation and administration of the complex dietary supplement (DSP) was described earlier [4], but the composition was adjusted (see: Table 1). Lutein and vanadium were added and dosages of four existing ingredients were adjusted. Preparation techniques were otherwise unchanged and palatability was not affected. As previously [51], dosages of each component reflect recommended daily human amounts (adjusted for mouse body size and higher metabolic rate).
**Lutein** was added (16.59mg/day/100mice; from Natural Factors®) to offset age-related vision loss. Lutein supports pigmentation for retinal protection against light-induced damage [52] and exhibits neuroprotective and antioxidant properties [53] (more detail in: Ch. 5).

**Vanadium** is a trace mineral commonly combined with chromium (roughly 1:4 ratio) in human dietary supplements. We used a Chromium&Vanadium supplement from Natural Factors®. Maintaining chromium concentrations to match the previous DSP composition [4,51] provided a dose of vanadium amounting to 0.08mg/day/100mice. The metabolic roles of vanadium and effects of its deficiency were described [54]. Toxic effects have not been reported [55]. Vanadium enhances insulin action and improves glucose tolerance [56,57]. The effects of our DSP on blood glucose are presented in [Ch. 5].

**Coenzyme Q10** was reduced from 60.89 to 24.84mg/day/100mice. A review of over 30 human studies on the clinical effects of CoQ_{10} suggests that substantial benefits can be achieved with moderated doses and relatively short treatment courses [58]. For daily benefits, high doses appear unnecessary. The present change reflects lowering the dose from the highest to medium recommended daily human intake. Still smaller concentrations were found to be effective [58].

**Rutin** dosage was reduced from 304.88 to 111.93mg/day/100mice. A higher dose was initially used as rutin absorption is slow in the rodent gut [59]. No toxicity was apparent at comparable doses via intravenous injections [60]. The present concentration is more comparable to daily human supplementation. Higher levels may simply increase bulk without added benefits.

**Acetyl L-Carnitine** concentration was elevated from 146.45 to 236.2mg/day/100mice. Recent findings reinforce the role of acetyl-L-carnitine in attenuation of behavioral and histological markers of Alzheimer’s symptomology [61]. The present dose falls in the median range recommended for aging humans.

A reduction of **Vitamin B6** (15%) was associated with switching to a new supplier. We do not anticipate any impacts resulting from this minor alteration.
**Behavioral studies:** *Locomotor intensity:* Mice were singly placed in an open field arena (56x56x40cm LxWxH) and videotaped from above for 5 minutes (40W white light illumination) (see: Fig. A1 in Appendix). Video recordings were later analyzed using EthoVision® image tracking software. Distance traveled and maximal running speed were scored.

*Wire hang:* Mice were allowed to grab onto a horizontal wire with both forepaws. Latency to fall onto a soft landing pad was recorded. A trial was discounted and repeated if a mouse managed to swing its tail around to grab the wire. Each mouse was tested three times with 10–20 minute inter-trial intervals on the same day.

*Knob hang:* Mice were allowed to grip onto a small (1.5cm diameter) metal knob protruding from a flat upright Plexiglas board. Maintaining a solid grip was challenging due to restricted movement and a somewhat awkward body position (see: Fig. A2 in Appendix). As before, latency to fall was measured and averaged over 3 same-day trials.

*Grip strength:* A mouse was held by base of tail and allowed to obtain a firm grip on a small wire handle which was connected to a weight resting on a flat-top electronic balance. The mouse was then slowly lifted until it released its grip. Readings from the balance were continuously recorded onto a computer. A diagram of the apparatus and further details are found in the appendix (Fig. A3). The three heaviest weight lifts achieved (peak force) were averaged. As smaller animals have less muscle mass, body size was taken into account so peak force is presented in mass-specific units (mN/g body mass).

**Tissue collection and assays:** Mice were decapitated; brains were removed and flash frozen in liquid nitrogen. Activity of mitochondrial complexes I and II was measured in brain homogenates as described in [28]. Levels of the damaging free radical, 4-hydroxynoneal, were assessed in whole brain homogenates and mitochondrial compartment as described in [28]. ATP concentrations were measured in homogenates from portions of the midbrain, cerebellum, pons and medulla using an APT colorimetric assay kit (BioVision Inc. Milpitas, CA, USA; Catalog #K354-100). Levels of hydrogen peroxide were measured in whole brain homogenates with the Amplex® red hydrogen
peroxide colorimetric assay kit from Molecular Probes®, Eugene, OR, USA (Catalog #A22188). Brains used for all assays were stored at -80°C.

**Resting body temperature:** Body temperature was recorded with a rectal thermometer during the light photoperiod. All mice were awake and mobile for at least 15 minutes prior to assessment. Room temperature was a constant 23ºC throughout.

### 4.4 RESULTS

**Locomotor activity:** Intensity of spontaneous locomotor activity was assessed by recording distance traveled and top running velocity\(^{19}\) in a dimly illuminated open field over a five minute period. Twenty four Nr and 32 Tg mice were tested. Gender effects were not resolved.

**Nr mice:** Distance traveled decreased as a function of age; however, a significant regression was only resolved for untreated Nr (p=0.02; Fig. 1). For either treatment group, distance traveled was \(~50\%\) less in 500 day old mice compared to 100 day old animals (Fig. 1). A significant effect of DSP treatment was resolved with ANCOVA (covariate=age). Travel distance of supplemented mice (in the time allowed) was consistently greater across a full range of ages, corresponding to an average increase of 39% in supplemented Nr mice compared to controls (p<0.05; Fig. 1). Despite a significant main effect of treatment, slopes (but not intercepts) of age-related regressions for supplemented and untreated animals were nearly identical (compare equations in: Fig. 1 caption). This suggests that impacts of the DSP on rates of decline were minor. Regardless, at any given age (including oldest animals), supplemented mice showed superior performance (i.e. greater travel distance: \(~39\%\) ) convincingly indicating that benefits of treatment extended to the oldest cohorts.

**Tg mice:** On average, Tg covered 40% less distance compared to age-matched Nr mice (ANCOVA, covariate=age; effect of genotype: p<0.0001) but no impact of the DSP on

\(^{19}\) Mean running velocity was scored but is not separately reported here. Since all trials lasted exactly five minutes, mean running velocity is entirely reflected in the distance traveled.
running distance was resolved. Effects of age were also poorly discriminated so Tg data were not graphically illustrated in Figure 1 to reduce visual obscurity.

**Top running velocity:** Maximal running velocity generally declined with age, but a significant age-related regression was only observed in untreated Tg mice. Both ANOVA and ANCOVA were applied to resolve differences between genotypes and treatment (nearly identical p-values were returned). Supplemented Nr had a significantly (16%) higher top running velocity compared to untreated controls (p<0.04; Fig. 2). Likewise, top speed was also higher in supplemented versus untreated Tg (20% difference; p<0.05; Fig. 2). Compared to Nr mice, top speed of Tg was 22% lower across all ages (ANOVA; effect of genotype: p<0.0002).

**Forepaw strength:** Forepaw strength of supplemented and untreated Nr and Tg mice was assessed on three tests. For each test a mouse was allowed three trials and the average data was used for analysis. To account for confounding effects of body size, the results are presented per unit of body mass. All tests indicated that forepaw strength was augmented by supplementation (Figs. 3&4). Mice were between 4 and 27 month old and no significant age-related effects were resolved, albeit forepaw strength was somewhat weaker in older mice.

**Wire hang:** Genotype effects were not resolved for the wire hang so Nr and Tg data were combined (n=62). Figure 3A shows the latency to fall when a mouse was suspended by the forepaws from a horizontal wire. Longer latency to fall corresponds with stronger grip. Supplemented mice showed a significant 108% increase in latency to fall (i.e. more than doubled) compared to untreated controls (p<0.05; Fig. 3A). This test may also reflect motor coordination [1] as mice were free to maneuver on the wire.

**Knob hang:** Instead of a wire mice were suspended from a small metal knob (to restrict horizontal movement, see: Appendix A2). Due to increased difficulty of this task, heavy transgenic mice were unable to hold on long enough to allow reasonable latency measures. Thus, only Nr mice were tested (n=22). Latency to fall was significantly higher (71% improvement) in supplemented mice compared to untreated controls (p<0.05; Fig.
3B). Latencies were substantially shorter compared to the wire hang test as the metal knob precluded a firm grip.

**Grip strength:** This protocol effectively measured the maximum weight (peak force) a mouse could lift using its front paws (see: Methods; and Fig. A3 in Appendix). Supplemented Nr mice were able to generate a 20% greater peak force (i.e. lift a heavier weight before releasing the grip) compared to untreated Nr (p<0.02; Fig. 4). Supplemented Tg mice showed a 7% increase but this was not significantly resolved (Fig. 4). Compared to Nr mice, Tg were able to lift a greater absolute weight; however, since Tg mice are nearly twice the size of Nr, mass-specific grip strength of Nr was actually 29% higher (p<0.0001; Fig. 4).

**Mitochondrial complexes I and II activity:** Complexes I and II (CI, CII) were assessed in brain homogenates of Nr mice only. Age-related trends for CI were not resolved. On average, supplemented Nr mice (n=11) showed a 32% increase in CI with age (from 3 to 22 months), while untreated Nr (n=10) showed a 20% decline (Fig. 5A). Overall, levels of CI activity were 14% higher (non-significant difference) in supplemented compared to untreated mice.

Treatment with the DSP had a strong effect on CII activity. Untreated mice showed a 31% decline in brain CII activity from 3 to 22 months of age (6.8 to 4.7 units), while supplemented Nr showed ~two-fold increases over the same age range (from 4.3 to 8.8 units) (Fig. 5B). The age-related positive regression of CII activity in the supplemented group was highly significant (p=0.003). Age-related regression lines were of opposite slope and were significantly differentiated (ANCOVA; covariate=age; slopes differ: p<0.001; Fig. 5B). In mice over one year of age, CII activity was doubled in supplemented compared to untreated mice (t-test: p=0.0004; Fig. 5C).

The effect of summing of CI and CII activity is shown in figures 5D and 5E. Both the age-related trends and mean values of old (>1 year) mice showed identical patterns to those of CII alone (compare Figs. 5B and 5D; and Figs. 5C and 5E). This indicates that effects of DSP treatment were entirely explained by CII alone while CI made little contribution to differences between treatment groups.
We graphed the relationship of CII with CI on a scatter-plot. Untreated mice showed a significant positive relationship ($r^2=0.897; p<0.001; \text{Fig. 5F}$). Conversely, supplemented mice showed a strong negative correlation (best approximated with a second order polynomial fit; Fig. 5F). The relevance of this finding is addressed in discussion.

**Brain ATP levels:** Levels of ATP in brain homogenates of Nr adult mice (18–24 months; n=20) were nearly identical irrespective of treatment (Fig. 6). At the oldest ages (>24 months; n=8), ATP levels in brains of untreated mice were significantly reduced (by 30%) compared to adult ages (ANOVA, post-hoc Duncan’s test: $p=0.045; \text{Fig. 6}$). However, old supplemented mice (>24 months) showed identical ATP levels to those of adults (Fig. 6) indicating complete prevention of declines in this critical cellular energy supply. This was further illustrated by a significant 34% difference between supplemented and untreated mice >24 months old (ANOVA, post-hoc Duncan’s test: $p=0.031; \text{Fig. 6}$). This difference entirely reflected loss of ATP in old untreated mice as opposed to any elevation in old supplemented mice.

Unlike the rest of the animals which appeared healthy, active and in fair body condition, one supplemented and one untreated mouse (ages: 25 and 30 months, respectively) were culled at endpoint. These mice had poor body condition (loss of body weight, frailty, low mobility, decreased appetite and poor hydration). If not euthanized, such animals expire within a few days. ATP levels of these endpoint mice were significantly lower ($p<0.01$) compared to all other animals suggesting that (irrespective of treatment) a drastic shortfall in brain ATP is experienced immediately prior to death (see: EP column in Fig. 6).

**Brain lipid peroxidation (4-hydroxynoneal):** Small reductions of 10–11% in levels of 4-hydroxynoneal (4-HNE) were detected in brain homogenates of supplemented Nr and Tg mice, respectively, compared to untreated controls, but this was not statistically resolved (Table 2). Supplemented Nr mice had a 19% reduction in 4-HNE levels in the mitochondrial compartment but this also fell short of significance (Table 2). 4-HNE in brain mitochondrial of Tg mice was not assessed at present.
Brian homogenate 4-HNE levels were positively correlated with age in Nr mice only (age range: 3–22 months; combined treatments: $r^2=0.29$; $p=0.015$; data not illustrated). Tg mice showed virtually no change with age and 4-HNE levels were not significantly higher than in Nr. Old (22 months) Nr mice showed a 45% increase in 4-HNE levels compared to young 3 month old mice. Age-related regressions were similar for both supplemented and untreated Nr.

**Brian hydrogen peroxide:** The most pronounced differences in brain homogenate $\text{H}_2\text{O}_2$ levels were gender-related. Only Nr mice were assessed ($n=37$; ages: 3–28 months; mean age=18 mo). Levels of $\text{H}_2\text{O}_2$ were 71% and 113% greater in males compared to females in the untreated and supplemented groups, respectively (ANOVA, post-hoc SNK: $p<0.001$; $p<0.0001$; Fig. 7A). DSP treatment had no impact on $\text{H}_2\text{O}_2$ levels in females (15% n.s. increase). However, supplemented males showed a significant 43% elevation in $\text{H}_2\text{O}_2$ levels compared to control males (Fig. 7A). Remarkably, despite a very wide range of ages, age-related trends were completely absent in either treatment group (Unt: $r^2=0.0116$, $p=0.64$; Supp: $r^2=0.0001$, $p=0.97$).

Assessment of mitochondrial complexes I–IV, protein carbonyls, 3-nitrotyrosine and 4-HNE reported here and previously [4,27,28] were carried out in different mice than those used for measure of $\text{H}_2\text{O}_2$ levels. We used the predicted values for each of complex I–IV, carbonyls, 3-NT and 4-HNE (from age-related regressions) to test for possible correlations with $\text{H}_2\text{O}_2$ levels. No significant regressions were found between any of these variables and levels of $\text{H}_2\text{O}_2$ (for probabilities see: Fig. 7B).

**Resting body temperature:** Resting body temperature was measured in supplemented and untreated Nr and Tg mice of both genders across ages (2–22 months; $n=591$). ANCOVA (covariate=age) was applied to test for main effects of genotype, sex, treatment and age; as well as higher order (two-way and three-way) interactions between: genotype*sex, genotype*treatment, sex*treatment and genotype*sex*treatment. ANCOVA found a strong significant main effect for each independent variable.

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20 3-nitrotyrosine (3-NT) is a measure or nytrosative protein damage [see: 28].
(summarized in Table 3). The two-way interaction between sex*diet was also significantly resolved (Table 3).

**Main effect of age:** Body temperature was negatively correlated with age when data for all mice was pooled (linear regression: $r^2=0.087; p<0.0001$). Mean body temperature of 3 months old mice was 37.7ºC compared to 36.7ºC for 22 months old mice (a drop of 1.0ºC).

**Main effect of genotype:** Mean body temperatures of Tg and Nr mice were 37.4 ºC and 37.8ºC, respectively (0.4ºC difference; p<0.00001).

**Main effect of gender:** Mean body temperature of male mice was 37.4ºC compared to 37.8ºC for female mice (0.4ºC difference; p<0.000001).

**Main effect of treatment:** Mean body temperature of supplemented mice was 37.8ºC compared to 37.5ºC for untreated mice (0.3ºC difference; p<0.0001).

**Higher order interaction effects:** While the main effect of diet was significantly resolved, consistent impacts of the DSP on temperature across genotypes and genders were unclear. Hence, effects of the DSP on body temperature were examined separately in each genotype and gender: Tg males, Tg females, Nr males and Nr females. Effects of treatment in each group were assessed using ANCOVA (covariate=age) post-hoc analysis with SNK test. Table 4 summarizes the results.

Diet had no detectable effect on body temperature of Tg male mice; however, significant impacts were found in both Tg and Nr females (p=0.009); and marginal significance was obtained in Nr males (p=0.083). In all cases (except for Tg males) the DSP elevated mean body temperatures (Table 4).

**Higher order interaction effects with age:** A strong main effect of age on temperature was obtained (Table 3). Hence, we examined whether treatment impacted the age-related trend in each genotype. To examine dietary impacts, we tested whether slopes of the age-related linear regressions differed with supplementation using the ANCOVA slope homogeneity test (combined sexes; regressions not shown graphically).

Both supplemented and untreated Tg mice showed non-significant temperature declines with age (linear regressions: p=0.36 and p=0.21; respectively). This corresponded to
~0.2°C drop from 3 to 18 months of age. Slopes of regression lines were not significantly different (post-hoc SNK: p=0.66).

On the other hand, slopes of linear regressions significantly differed between supplemented and untreated Nr (ANCOVA post-hoc SNK: p<0.03). This indicates that the DSP significantly altered age-related changes in body temperature in Nr mice. Specifically, untreated Nr mice showed a decline in temperature with age (linear regression: $r^2=0.126$; p<0.0001) corresponding to a drop of 0.9ºC from 3 to 22 months of age. Conversely, supplemented Nr mice showed no age-related relationship ($r^2=0.004$). This indicates that DSP treatment prevented the age-related declines in resting body temperature in Nr mice, effectively maintaining youthful body temperature into oldest ages.

4.5 DISCUSSION

**Motor function in aging:** We previously reported that daily locomotor duration was elevated in old supplemented mice compared to age-matched controls [4]; however, it remained unknown whether intensity was also affected. Present behavioral data show that supplemented mice indeed maintain stronger physical intensity into older age. Senescent rodents generally express lower running speeds [1,8], reduced running distance [1,9] and diminished motor coordination on tests of horizontal wire suspension and rotarod balance [1,10]. We previously showed that DSP treated aging mice had improved motor balance on the rotarod [Ch. 3]. Here we document faster running speed, greater distance traveled, and longer fall latency on the horizontal wire hang (Figs. 1,2,3A). Bradykinesis (impaired locomotor function) is common to virtually all aging animals [1–6] and is associated with diverse age-related conditions [4]. Hence, the present findings document strong amelioration of functional aging by dietary supplementation.

Declining motor skills are closely associated with elevated markers of protein damage (carbonyls) in specific regions of the CNS [1] and throughout the brain [4]. The DSP significantly reduced oxidative [4] and nytrosative [28] protein damage in brains of aging mice. This was associated with elevated levels (duration) of spontaneous locomotion [4],
spontaneous exercise duration [Ch. 5] and intensity of locomotion (Figs. 1, 2). We also show here that brain mitochondrial function (complex II; Figs. 5B, C) (and previously complex III [4]; and complex IV [27]) was boosted in old supplemented mice and that significant declines in brain ATP levels were completely prevented in the oldest (>2 year old) mice (likely comparable to humans >70 years of age). Central brain alterations appear strongly associated with motor functioning of aging mice.

**Aging muscle:** While many age-related motor impairments are linked to central neuronal and synaptic changes [3, 62, 63], degeneration of skeletal muscle function and muscle loss also contribute [3, 11–15, 19]. Muscular deficits must ultimately limit execution of even simple motor tasks such as walking [3, 63, 64]. Similar to the brain, aging muscle also shows elevated accumulation of DNA, lipid and protein damage [21, 65]. Given the high metabolic rate and high ROS production in brain and muscle, these tissues are thought to be particularly susceptible to oxidative damage with age [2].

We did not assess oxidative/nitrative damage markers in muscle so we cannot directly address possible protective effects of the DSP at the level of skeletal muscle cells. However, grip strength was significantly augmented in DSP treated mice (Figs. 3, 4); and grip strength is a standard biomarker of muscle function and age-related decline [20–23]. In humans, protein carbonyl concentrations in muscle are negatively associated with grip strength [21, 23]. Aging persons show weak grips, and oxidative damage is thought to be directly contributing [21]. Lower grip strength is also a reliable marker of metabolic syndrome associated with type 2 diabetes and general sarcopenia (age-related loss of muscle mass) [22]. Declining grip strength closely predicts all-cause mortality in humans [20]. In fact, skeletal muscle strength appeared to be a better independent predictor of mortality than muscle mass or physical activity levels [20].

**Sarcopenia and physical activity:** Increased skeletal muscle strength in DSP treated mice is likely a major factor contributing to faster and longer locomotor activity. There is overwhelming evidence that regular physical activity can prevent a variety of age-related pathologies and premature mortality [reviewed in 17]. Furthermore, reversal of some age-related conditions is possible when inactive persons adopt active lifestyles [16, 17].
Significant health improvement may be attained by following relatively undemanding activity regimes [17] such as brisk walking for 20 minutes per day [18]. However, many elderly persons may be unable to adopt these regimes due to loss of skeletal muscle (sarcopenia).

According to some reports, prevalence of sarcopenia may be as high as 24% in persons 65 to 70 years of age and exceed 50% in those over 80 years old [66]. Persons with sarcopenia have severe walking limitations and may require assistive devices [14]. When progressed, sarcopenia is very difficult to reverse although some improvement with resistance exercise is possible [13]. Sarcopenia is generally diagnosed when the appendicular muscle mass is more than two standard deviations below the mean value of a younger reference group [66]. Lauretani et al [15] found that grip strength accurately reflected muscle loss and could be used to correctly diagnose sarcopenia using the same standard deviation criteria. Given the present grip strength data, and providing that effects translate to humans, DSP treatment is expected to considerably slow or delay onset of sarcopenia. Supplementation could allow more of the elderly population to engage or maintain active lifestyles and obtain the associated health benefits. Other limitations, such as cardiovascular function, must ultimately be considered, although the ability of the cardiovascular system to adapt to endurance exercise is not as affected by aging as other possible limiting factors [16].

**Mitochondrial complexes:** The present work shows impacts of the DSP on activity of mitochondrial complexes I and II in brains of aging mice. Complexes I, III and IV (CI, CIII, CIV) were initially of greater interest as they participate in proton translocation (i.e. direct link to energy production) [41], are the major sites for ROS generation (particularly I and III), and are implicated in aging and several age-associated pathologies including neurodegenerative diseases [4,27,33]. In addition, CI, CIII and CIV contain domains encoded by mtDNA [42,67,68] where higher mutations rates are expected. On the other hand, mammalian complex II (CII) is entirely nuclear encoded, it has no proton pump function and does not generate ROS under normal conditions [41,69]. CII-associated diseases are relatively rare (usually inborn mutations with wide clinical consequences).
and implications in general aging are unclear [41,44,70]. Hence, CII was not closely considered in previous work. Previous analysis of CI activity revealed no effects of DSP treatment. Age-related trends were weak and data showed high variability (Fig. 5A). However, the DSP strongly impacted CIII and CIV activity that was closely associated with motor and cognitive improvements in old age [4,27].

In conjunction with new findings presented here, we re-examined impacts of the DSP on CI and CII activity. The role of complex II deficiency in Huntington’s disease [33,71–73] and tumor formation [44,70,74,75] highlights the possible importance of this enzyme. Furthermore, it appears that alterations in CII (which are often overlooked [41,43]) may directly contribute to ROS generation under hypoxic conditions [43] or when defective [69,74]. CII also regulates ROS production at CI via modulation of substrate (NADH) availability, or by reverse electron transport [43,76].

We previously reported that activity of mitochondrial CIII and CIV in brain followed expected declines with age in Nr control animals [4,27]. Remarkably, DSP treated mice showed a strongly increasing age-related trend such that in old age (~2 years) CIII and CIV activities were ~85% higher in supplemented mice compared to age-matched controls [4,27]. Surprisingly, CI activity was unaltered by the DSP (Fig. 5A) and correlations between CI activity and activities of CIII and CIV were entirely absent ($r^2=0.026$ and $r^2=0.002$, respectively). The question regarding the source of electrons to support the marked boost of CIII and CIV remained unanswered. Here we considered whether CII may be contributing.

We show that activity of CII declined with age in the untreated group, but increased in supplemented animals ($r^2=0.635$; $p=0.003$; Fig. 5B). In supplemented mice older than one year, CII activity was two-fold higher compared to age-matched controls ($p=0.0004$; Fig. 5C). Further, CII activity expressed age-related patterns identical to CIII and CIV according to DSP treatment (compare Fig. 5B with Fig. 3A in Ch. 1 and Fig. 2 in Ch. 2).

Complex II is involved in the Krebs cycle (catalyzing oxidation of succinate to fumarate) and in the ETC, where it couples energy from succinate oxidation to reduction of ubiquinone to ubiquinol (the Q-pool) [41–43]. The electrons gained by the Q-pool are
passed along to CIII and CIV and ultimately to molecular oxygen [42–44]. NADH molecules produced in the Krebs cycle (reduction of NAD+ to NADH) also transfer electrons to ubiquinone. This reaction is catalyzed by CI, and is the chief entry point for electrons into the mitochondrial ETC [41]. Unlike CII, electron transfer at CI is coupled to proton translocation [41,44].

In this fashion, both CI and CII initiate the first step in the mitochondrial respiratory chain, although the relative contribution of CI is often greater. It is important to emphasize that electrons entering the ETC via CII bypass CI entirely [41,42,44]. As a result protons are not exchanged, ROS are not generated, but reduction of the Q-pool (i.e. electron transfer to ubiquinone) still takes place. In this fashion, upregulation of CII activity can provide electrons to CIII and CIV irrespective of CI status. This mechanism is that most consistent with our findings, since we did not observe any impact on CI activity, but complexes II–IV were all significantly upregulated in old DSP treated mice.

Graphing the sum of CI+CII activity with respect to age in supplemented mice does not improve (or hinder) the age-related correlation of CII alone (CII: $r^2=0.635$, p=0.003; CI+CII: $r^2=0.624$, p=0.004; Figs. 4B,D). CI+CII activity in old (>1 year) supplemented mice was exactly twice that of untreated animals (Fig. 5E), a difference entirely reflected by CII alone (Fig. 5C). These findings clearly indicate that effects of DSP treatment pivot entirely on CII upregulation. Since CII is not coupled to proton translocation, CIII and CIV are obviously required to drive the electrostatic gradient; however, their activity is ultimately reliant on Q-pool reduction.

Evidence for discriminatory compensation of complex II in DSP treated mice: An interesting observation pertains to the apparent ‘discriminatory’ modulation of CII activity with respect to CI status in conjunction with DSP treatment. The scatter-plot of CI activity with age (Fig. 5A) shows a wide range of values. CI activity in the five oldest (>10 months) supplemented mice varied between 1.8 and 6.3 units (i.e. the entire length of the y-axis). At the same time, these mice were tightly packed within a narrow vertical margin on the CI+CII age-related scatter-plot (Fig. 5D). This suggests that old supplemented mice experienced different degrees of CII upregulation which appears to
depend on activity of CI. To test this, we graphed the relationship between CI and CII in old (>10 months) mice (Fig. 5F). A clear negative relationship between CII and CI activity emerged in the supplemented group (Fig. 5F). Mice with high CI activity showed low levels of CII, whereas mice with low CI activity showed highly elevated CII levels. It appears that CII activity did in fact depend on CI activity levels, such that the end result was essentially identical with respect to combined activity of CI+CII (i.e. balanced to a set point).

We must note that activity of neither ETC complex was actually controlled in our study; hence we may not infer causality of the negative relationship between CI and CII in supplemented mice. However, in terms of a unifying mechanism, it is most probable that CII ‘steps up’ when CI is deficient, as opposed to the contrary. Kruse et al [77] showed that mice with complete loss of CI function attained normal CIII and CIV activity and normal ATP levels in the first 35 days of life via upregulation of CII. Mice with partial loss of CI were indistinguishable from wild type for the duration of study [77]. This suggests that considerable compensatory capacity of CII is possible. Conversely, inhibition of CII is poorly tolerated [71]. Theoretically, ETC action may continue despite CII inhibition provided that NADH is available for CI [44]. However, because CII also participates in NADH synthesis (Krebs cycle) [41,42,44], this scenario is unlikely. Although, if only the ubiquinone reductase domain of CII is damaged, while the succinate oxidase domain is intact, renewal of the NADH pool will go on and electron transfer to ubiquinone can proceed exclusively via CI. The latter, of course, is entire incidental upon damage to specific domains. Hence, the observed negative relationship between CII and CI (Fig. 5F) mostly plausibly resulted from compensation by CII when CI levels were low.

Interestingly, age-matched untreated mice were tightly locked into a positive correlation between CII and CI ($r^2=0.897$; Fig. 5F); and both CI+CII were negatively correlated with age (Fig. 5D). This suggests that compensatory ability by CII becomes lost or that CII was a limiting bottleneck. CI and CII activity levels in platelet mitochondria from aging humans were also positively correlated with each other and with
ubiquinone (coenzyme Q<sub>10</sub>)<sup>21</sup> [78]. At this point further investigation is necessary and mechanisms remain unclear, but the highly significant differences and completely opposite trends certainly point to strong impacts of the DSP. However caused, CII upregulation with age was associated with boosted CIII and CIV activity [4,27], higher ATP levels (Fig. 6), augmented physical function (Figs. 1–4; [4, Ch. 5]), neuroprotection [Ch. 3,6] and superior cognitive ability [27; Ch. 6] in our mice.

CII deficiency in striatal neurons is associated with Huntington’s disease [72,73]. Given the highly elevated CII levels in brain mitochondrial and higher counts of healthy neurons in striatum of old supplemented mice [Ch. 6] it appears the our DSP may emerge as a particularly powerful treatment if impacts translate to humans [see: 28].

**Regulation of ATP production:** Elevated activity of ETC complexes III and IV (such as in old DSP treated mice [4,27]) should create a stronger proton gradient across the inner mitochondrial membrane. This suggests that ATP production by ATP synthase (complex V) may be greater, unless mitochondria are also highly uncoupled. Neither ATP synthase activity nor density of uncoupling proteins were measured, hence we are unable to argue that rates of ATP synthesis were elevated. In fact, body temperature of supplemented mice was higher than controls (Tables 3&4) which may indicate higher uncoupling (or elevated metabolic rate). Of course other factors may have greater impact on temperature regulation, and obviously our data reflects only brain mitochondria (i.e. limited contribution to whole-body thermal status).

Brown [34] reviews a wide array of mechanisms controlling ATP synthesis in mammalian cells. The entire regulatory system is rather complex: technically, all modulators of pathways limiting substrate availability for cellular respiration and ATP synthesis can be involved, as well as rates of ATP diffusion and utilization [34]. A detailed review is beyond the scope of the present work; however general principles relevant to present findings are synthesized below.

Early works by Chance and Williams [79,80] showed that rates of ATP production were ultimately regulated by demand (i.e. ATP usage). ATP synthase activity is sensitive

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21 Coenzyme Q<sub>10</sub> is included in our DSP.
to ATP, ADP and P_i levels [81]. As cytoplasmic ATP falls, more ADP (and P_i) is liberated and transported to the mitochondrial matrix in exchange for ATP. This increases substrate availability for oxidative phosphorylation which stimulates a mass action effect down the entire respiratory chain [see: 34]. Higher proton influx through complex V (ATP synthase) decreases the potential across the inner membrane which was shown to boost activity of complexes IV, III and possibly I [82–84]. In this fashion, unless oxygen or NADH levels are limiting (or ETC enzymes are impaired), elevated energy demand is met by rapid ATP production. Similarly, when demand falls, the higher ATP/ADP ratio will diminish rates of ATP synthesis. In either case, cellular ATP stores remain remarkably constant.

**Dietary treatment and ATP levels in aging mice:** ATP levels measured in brain homogenates of adult mice were equal between treatment groups (Fig. 6). This does not imply similar rates of ATP production or utilization, but suggests that energy demands (whatever they may have been) were sufficiently met by oxidative phosphorylation. Increased activity of ETC (complexes II–IV) and higher physical activity of supplemented mice (Figs. 1, 2; [4] and [Ch. 5]) may indicate increased ATP turnover (yet to be examined).

ATP levels of old supplemented mice (>2 years) were identical to younger adults; but age-matched (old) untreated animals showed significant decreases in ATP compared to younger mice (30%, p=0.045) and to old supplemented mice (34%, p=0.031; Fig. 4). This convincingly suggests that old untreated mice experience significant energy shortfalls in brain while DSP treated mice did not. In untreated mice ATP deficiency was undetectable prior to 2 years of age, suggesting that energy deficits in brain directly contribute to aging as mice quickly deteriorate thereafter and rarely survive past 2.5 years. Additional support is evidenced in mice with endpoint body condition. Brains from two mice were harvested at age-related endpoint (loss of body weight, frailty, immobility, decreased appetite and poor hydration; ages: 25 and 30 mo). These mice had especially low ATP levels (EP column in Fig. 6). It appears that mice experience a sharp plummet in brain
ATP a few days leading up to death. Such findings must be interpreted against the normal strong homeostatic control of ATP production in youthful physiology.

ATP deficits in old untreated mice likely result from oxidative damage to respiratory chain enzymes [31] (or reduced mitochondrial turnover [30]). For example, CII inhibition results in rapid ATP decline despite available substrates [71]. Interestingly, significant reductions in activity of complexes II–IV in untreated mice preceded ATP shortage by several months\(^22\). This may indicate some residual capacity to sustain ATP balance (perhaps by upregulating glycolysis) or that energy demands rose higher with further aging. In astrocytes, the contribution from glycolysis to ATP synthesis is relatively minor (up to 32\%) compared to oxidative phosphorylation [35] and even less so in neurons [40]. In addition, neurons do not show a capacity to invoke glycolysis for sustained ATP production when oxidative respiration is depleted [39,85]. Hence, considerable neurodegeneration is expected when ETC complexes fail. Indeed, cognitive and motor impairments as well as neuronal loss was evident as early as 1.5 years in untreated mice [4,27; Ch. 3,6] indicating that substantial damage was sustained prior to explicit ATP shortfalls. The importance of strong mitochondrial function with respect to neurodegenerative diseases such as Parkinson’s, Alzheimer’s and Huntington’s was previously highlighted in the context of possible benefits of the DSP [4,27,28]. Enhanced mitochondrial activity should offset age-related energy shortfalls and the present data suggest this is substantial.

In human brain, ATP levels can drop by \(~25\%\) at old age, but similar manipulations in vitro were not immediately reflected in reduced cell vitality [29]. Instead, this resulted in forestalled DNA repair activity which eventually contributed to wider damage [29]. This may explain why our untreated mice do not immediately deteriorate and survive for some months on low ATP, presumably accumulating DNA damage at increasing rates. DNA repair defects can cause premature aging phenotypes [86]. Therefore, DNA repair, or rather lack thereof, is directly contributing to aging. Higher ATP levels in old

\(^{22}\) Compare ages of untreated Nr mice with low complex II activity (Fig. 5B); low complex III activity (Fig. 3A in Ch. 1); and low complex IV activity (Fig 2 in Ch. 2) with ages of mice (>2 y) showing low ATP levels (Fig. 6).
supplemented mice could be expected to facilitate more efficient DNA repair and slow aging.

The major consumers of ATP in brain are the Na\(^+/\)K\(^+\) ATPase and protein synthesis [34,35]. Therefore, these systems will likely suffer most when energy runs low, or conversely, completely drain energy from other vital functions (e.g. DNA repair, antioxidant defenses). Dysfunction of the Na\(^+/\)K\(^+\) ATPase pump is associated with neurological disorders and neurodegeneration such as Alzheimer’s [36–38]. Higher ATP availability in old supplemented mice is expected to reduce the risk of developing neuronal loss. In fact we reported that DSP treatment was associated with higher neuronal numbers in brain regions implicated in cognitive and motor aging [Ch. 3,6]. Protein synthesis, shown to decline in brains of aging rodents [87], may also be rescued through increased energy availability. Clearly, the benefits of adequate energy supply have the widest possible implications in aging.

**Cost of high oxidative respiration:** General conventions hold that costs of elevated oxidative phosphorylation are higher ROS generation and higher oxidative damage; however encouraging exceptions are not uncommon [88,89]. Expectedly, the relationship between mitochondrial activity and protein carbonylation was strongly positive in untreated mice [4]. We presently found that brain H\(_2\)O\(_2\) levels were significantly higher in supplemented Nr males (females were not resolved; Fig. 7). Despite elevated H\(_2\)O\(_2\), markers of lipid peroxidation (4-HNE) were not elevated in whole brain homogenates nor in the mitochondrial compartment of supplemented mice (Table 2). Furthermore, oxidative and nytrosative damage was actually reduced in DSP treated mice and correlated negatively with increased mitochondrial activity [27,28]. DNA damage markers were also lower in supplemented mice [47]. Correlations of H\(_2\)O\(_2\) with age were completely absent in all mice (\(r^2\)=0.0057); and more importantly, H\(_2\)O\(_2\) levels did not correlate with activity of any ETC complexes, carbonylation, nitration or lipid peroxidation (see: Fig. 7B). These findings indicate that (a) H\(_2\)O\(_2\) did not predict cellular damage in our mice; and (b) H\(_2\)O\(_2\) levels were independent of mitochondrial function. Instead, extra-mitochondrial sources may be contributing (e.g. NADPH oxidases [50]). In
fact, the contribution of NADPH oxidase to free radical generation may surpass other sources [50]. H$_2$O$_2$ has mandatory roles in cell-signaling pathways [48,49,90], thus higher levels in supplemented animals may simply reflect augmented cellular activity.

**Resting body temperature:** Some animals with exceptional 35 year lifespans (such as pigeons) operate at high body temperature (~39.7–42.6°C; [91]) compared to equal-sized mammals (e.g. rat; longevity: ~3 years; body temperature: ~37.0–38.0°C [92]). Longevity of pigeons is not attributed to body temperature per se, but thought to result from low rates of free radical production despite elevated oxygen consumption and mitochondrial function [88,89]. The temperature elevation is likely a by-product of high oxidative metabolism. Our DSP treatment elevated body temperature of females and Nr, but not Tg, males (Table 4). Mitochondrial function (though only assessed in brain) was higher in supplemented mice (Fig. 5; [4,27]) while oxidative damage was unchanged (Table 2) or reduced [4]. Longer lifespan in Nr (11%) and Tg mice (28%) was also reported [93]. While the resemblance of supplemented mice is small in magnitude, it is directionally consistent with the pigeon phenotype (i.e. increased mitochondrial function, temperature and longevity, but decreased oxidative stress). However, the relevance of this comparison is somewhat questionable (see below).

It is interesting then, that in mammals (rodents and primates), increased longevity is associated with reduced body temperature [94]. This pertains to intra-specific correlations as opposed to between species, although the latter is also usually true. Long-lived calorically restricted rats and rhesus monkeys have lower body temperature compared to ad libitum fed controls [95,96]. Long-lived dwarf mutant mice also display reduced temperature [97]. At the same time, short-lived senescence-accelerated mice such as our growth hormone transgenics also have low body temperature and poor tolerance to cold stress [98]. No linear correlation is possible here. It appears that the high cost of thermoregulation is traded off in favor of high growth (e.g. Tg mice [98]) or in favor of longevity (caloric restriction or dwarf mice) [95,97].

Collectively, it is clear that body temperature per se does not predict nor influence longevity. In addition, across-species comparisons (such as the earlier pigeon/rat
example) may not be entirely useful as a wide range of other phenotypic traits aside from oxidative metabolism may have greater impacts (e.g. behavior, insulation, vascular anatomy, diet, endocrine factors, etc). In the case of our mice, it is likely that DSP-induced temperature differences reflect higher oxidative metabolism, however oxygen consumption studies are needed to confirm this. Limited preliminary data (unpublished) suggests that oxygen consumption may indeed be higher in supplemented mice.

**Summary:** We show for the first time that age-related decline in mitochondrial respiratory chain enzymes in brains of our old mice was associated with significant ATP shortage in old age. Remarkably, this was entirely prevented by treatment with a complex dietary supplement: ATP levels of the oldest supplemented mice were identical to those of young animals. This was associated with increased physical intensity across ages and stronger muscular function as found with the forepaw grip test (a biomarker of sarcopenia). High activity of respiratory complexes III and IV in old supplemented mice [previously reported: 4,27] was associated with increased complex II, but not complex I activity. Despite higher mitochondrial function and higher H$_2$O$_2$ levels in brains of supplemented mice, lipid peroxidation (i.e., damage) was unchanged. Elevated H$_2$O$_2$ may have reflected extra-mitochondrial sources and increased cellular signal transduction activity. Body temperature of supplemented mice was elevated, possibly reflecting boosted oxidative phosphorylation. These – and previous findings [4,27,51; Ch. 3,5] suggest great promise for slowing functional aging in humans via complex dietary supplementation.

4.6 ACKNOWLEDGMENTS

Authors recognize the contribution of Sarthak Matravadia and Zoya Tov in assistance with behavioral studies and diet preparation. We thank Dr. Henry Szechtmans for providing the image tracking system. Research funding supported by a grant to C.D. Rollo (NSERC).
4.7 FIGURES and TABLES

Table 1. Ingredients and dosages of the DSP

<table>
<thead>
<tr>
<th>Component</th>
<th>Dose (mg/day/100 mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₁</td>
<td>30.49</td>
</tr>
<tr>
<td>Vitamin B₃</td>
<td>30.49</td>
</tr>
<tr>
<td>Vitamin B₆</td>
<td>51.76 ↓</td>
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<tr>
<td>Vitamin B₁₂</td>
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<tr>
<td>Vitamin C</td>
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<tr>
<td>Vitamin E</td>
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<tr>
<td>Acetyl L-Carnitine</td>
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<td>Alpha-Lipoic Acid</td>
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<td>ASA</td>
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<tr>
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<td>Bioflavonoids</td>
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<td>Cod Liver Oil</td>
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<tr>
<td>CoEnzyme Q₁₀</td>
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</tr>
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<td>Flax Seed Oil</td>
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<tr>
<td>Folic Acid</td>
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<td>L-Glutathione</td>
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<tr>
<td>Lutein</td>
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<tr>
<td>Magnesium</td>
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<tr>
<td>Manganese</td>
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<td>Melatonin</td>
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<td>N-acetyl Cysteine</td>
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<tr>
<td>Potassium</td>
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<tr>
<td>Rutin</td>
<td>111.93 ↓</td>
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<tr>
<td>Selenium</td>
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<tr>
<td>Vanadium</td>
<td>0.08 *</td>
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</table>

Formula slightly differs from previous works [4–6]
*newly added; ↑dose increased; ↓dose lowered
All changes addressed in Methods

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Table 2. Levels of 4-hydroxynoneal (4-HNE) (optical scan: relative density units) in brain homogenates of Nr and Tg mice, and in brain mitochondrial compartment of Nr mice. Supplemented Nr mice had a 10% and 19% reduction in 4-HNE levels in brain homogenate and mitochondrial compartment, respectively, compared to untreated controls. Supplemented Tg mice had an 11% reduction in brain homogenate 4-HNE compared to Tg controls. In all cases differences between treatments fell short of statistical resolution. (Nr: n=21; Tg: n=19; ages: 3–22 months)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Brain homogenate ±SE, [N]</th>
<th>Brain mitochondria ±SE, [N]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Untreated</td>
<td>2360.7±192.2 [10]</td>
<td>4410.6±672.0 [11]</td>
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<tr>
<td>Transgenic</td>
<td>Untreated</td>
<td>2491.6±241.4 [8]</td>
<td></td>
</tr>
<tr>
<td>Transgenic</td>
<td>Supp</td>
<td>2215.1±205.8 [11]</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Effects of independent variables on resting body temperature in mice analyzed with ANCOVA. Total n=591 mice; ages: 2–22 months

<table>
<thead>
<tr>
<th>Variable</th>
<th>ANCOVA</th>
</tr>
</thead>
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<tr>
<td>Age</td>
<td><strong>p&lt;0.00001</strong></td>
</tr>
<tr>
<td>Genotype</td>
<td><strong>p&lt;0.00001</strong></td>
</tr>
<tr>
<td>Sex</td>
<td><strong>p&lt;0.00001</strong></td>
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<tr>
<td>Treatment</td>
<td><strong>p=0.00008</strong></td>
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<tr>
<td>Genotype*Sex</td>
<td>p=0.826</td>
</tr>
<tr>
<td>Genotype*Treatment</td>
<td>p=0.821</td>
</tr>
<tr>
<td>Sex*Treatment</td>
<td><strong>p=0.010</strong></td>
</tr>
<tr>
<td>Genotype<em>Sex</em>Treatment</td>
<td>p=0.118</td>
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</table>
Table 4. Effects of treatment on body temperature were compared in Tg and Nr male and female mice (n=591). Mean values averaged across ages are shown to illustrate overall effects. However, because age had a strong effect on body temperature (see: Table 3), statistical testing was done with 3-way ANCOVA (covariate=age; post-hoc SNK). Significant differences in bold. Ages: 2–22 months.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Mean Body Temp. (°C)±SE</th>
<th>Diff. (°C)</th>
<th>SNK</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Untreated</td>
<td>Supplemented</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transgenic</td>
<td>Male</td>
<td>37.30±0.04</td>
<td>37.26±0.09</td>
<td>0.04</td>
<td>p=0.735</td>
</tr>
<tr>
<td>Transgenic</td>
<td>Female</td>
<td>37.41±0.06</td>
<td>37.84±0.17</td>
<td>0.43</td>
<td><strong>p=0.009</strong></td>
</tr>
<tr>
<td>Normal</td>
<td>Male</td>
<td>37.52±0.08</td>
<td>37.76±0.15</td>
<td>0.24</td>
<td>p=0.083</td>
</tr>
<tr>
<td>Normal</td>
<td>Female</td>
<td>37.89±0.07</td>
<td>38.19±0.10</td>
<td>0.30</td>
<td><strong>p=0.009</strong></td>
</tr>
</tbody>
</table>
Figure 1. Distance traveled in dim open filed (5 min trial) by Nr supplemented and untreated mice with respect to age (n=24). Linear regressions:
Unt: \( y=29.10-0.03x; r^2 = 0.37; \textbf{p=0.020} \)
Supp: \( y=35.64-0.04x; r^2=0.15; \textbf{p=0.278} \)
A significant effect of treatment was resolved with ANCOVA (covariate=age; \textbf{p<0.05}). Mean distance traveled averaged across ages was 39% greater for supplemented compared to untreated mice. Animals in both groups showed a ~50% decline from 100 to 500 days of age. (Tg data not shown)
Figure 2. Maximal running velocity of mice in dim open field. Velocity generally decreased with age; however, significant correlations were only resolved for untreated Tg mice. Hence data are presented as group mean values for visual simplicity. Statistical analyses were done both with ANOVA and ANCOVA (covariate=age) and effects of genotype*treatment were resolved with post-hoc SNK test. Both statistical tests yielded significant differences. A 16% increase in top running velocity was seen in supplemented Nr (p<0.04), and a 20% increase was seen in supplemented Tg mice (p<0.05) compared to respective controls. Overall, Tg had a lower top velocity compared to Nr mice (ANOVA; effect of genotype: p<0.0001)
Figure 3. (A) Wire hang test: Effects of genotype were not resolved; hence Nr and Tg were pooled. Latency to fall was 108% greater for supplemented mice compared to untreated controls (n=62; t-test: *p<0.05). (B) Knob hang test: This test proved too challenging for Tg, thus only Nr mice were assessed. Latency to fall was 71% greater for supplemented compared to untreated mice (n=22; t-test: *p<0.05). For both (A) and (B) ages ranged from 4–27 months, no age related effects were resolved. Latency to fall was recorded and averaged over 3 consecutive trials.
Figure 4. Grip strength (front paws) was assessed by recording mass specific peak force (Nr n=46; Tg n=32; ages: 4–27 months, no effects of age were found). Grip strength was 29% greater in Nr compared to Tg mice (t-test: $p<0.0001$). Supplemented Nr mice showed a 20% stronger grip compared to untreated controls (ANOVA; post-hoc SNK: *$p<0.02$). A 7% increase in grip strength of supplemented transgenic mice was not significantly resolved.
Figure 5. See caption on next page
Figure 5. (Previous page) Brain mitochondria of Nr mice (untreated n=9; supplemented n=11; no gender effects; ages: 3–25 months). (A) Activity of complex I was unaffected by age or the DSP. Weak regressions were not resolved but age-related trends overall resembled those of complex II. (B) Untreated mice showed a 31% decline in complex II activity ($r^2=0.414$; $p=0.062$), while the supplemented mice showed a 105% increase in complex II ($r^2=0.635$; $p=0.003$) from 3 to 22 months of age. Slopes of age-related regressions were significantly differentiated with ANCOVA (covariate=age; $p<0.001$). (C) Complex II activity in mice >1 year old: supplemented mice showed a twofold elevation compared to untreated controls (t-test: **$p<0.001$). (D) Summed activity of complexes I+II showed similar age-related trends to those of complex II alone. Age-related regression for supplemented mice was resolved ($r^2=0.624$; $p=0.004$) and slopes were opposite and significantly different (ANCOVA; covariate=age: $p<0.005$). (E) Summed activity of complexes I+II was twofold higher in supplemented mice (t-test: *$p<0.01$). (F) Relationship between complex II and complex I activity in untreated and supplemented mice (>10 months old; n=11). Untreated mice showed a strong positive relationship ($r^2=0.897$; t-test: *$p=0.0004$). Supplemented mice showed a negative polynomial relationship
**Figure 6.** ATP levels in brain homogenates of healthy untreated and supplemented mice. Adult ages: 18–24 months (n=20) and old: >24 months (n=8). Two mice (one from each treatment) were sacrificed at end-point (EP) at ages of 25 and 30 months. ATP levels in brain of EP mice were significantly lower compared to adult mice (**p<0.01**). Levels of ATP were nearly identical in brains of adult mice irrespective of treatment. Untreated old mice showed a 30% decline is ATP compared to untreated adult mice (ANOVA, post-hoc Duncan’s test: *p=0.045*). Supplemented old mice showed no decrease from adult values, and had 34% higher ATP compared to untreated old animals (ANOVA, post-hoc Duncan’s test: *p=0.031*)
Figure 7. (A) Levels of hydrogen peroxide in brain homogenates of supplemented (n=15) and untreated (n=22) Nr mice. Untreated males had 71% higher H$_2$O$_2$ levels than untreated females (**p<0.001), and supplemented males had 113% more H$_2$O$_2$ than supplemented females (**p<0.0001). Supplemented males had 43% more H$_2$O$_2$ compared to untreated males (*p<0.03). A 15% H$_2$O$_2$ elevation in supplemented compared to untreated females was not significantly resolved (p=0.20). Mice were between 3–30 months; age-related trends were entirely absent. Groups were compared with ANOVA, post-hoc SNK test. (B) Linear correlations (R$^2$ and p-value) between brain H$_2$O$_2$ and brain mitochondrial complexes I–IV, 4-hydroxynoneal (4-HNE), protein carbonyls and 3-nitrotyrosine (3-NT). Values for ‘Variable’ (column 1) we predicted based on age related liner regression as previously done [see: 27]. No correlations between H$_2$O$_2$ and any of the variables was established.
4.8 REFERENCES


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4.9 APPENDIX

**Figure A1.** Open field (and mouse) used to assess distance traveled and running velocity (five minute trials); photograph taken in normal room illumination. Actual testing was done in dim 40W light to reduce anxiety. Videotaped from above with an infrared camera.

**Figure A2.** Knob-hang experiment: showing mouse grabbing onto a metal knob protruding from an upright Plexiglas board. Notice the restricted body position. This prevented mice from using hind paws and tail to aid in maintaining grip. Latency to fall was considerably shorter compared to the horizontal wire-grip test. Heavy Tg mice were unable to perform on this task.
Figure A3. Diagram of the grip strength apparatus. A mouse was suspended by the base of the tail and allowed to grab onto a wire handle with front paws (A). The wire handle was attached via a non-elastic connector to a 500g weight resting on a top-balance platform. Once the mouse got a firm grip it was slowly lifted up which decreased the reading on the scale (B). The reading from the scale was continuously recorded by a computer at 0.5 second intervals. Mouse was lifter until it released the grip. The lowest reading on the scale in (B) was subtracted from the 500g weight in (A). This difference corresponded to the heaviest weight lifted (peak force) during a single trial. Three consecutive trials were averaged.
CHAPTER 5

Chapter introduction:

The major findings presented in this chapter illustrate impacts of our DSP on blood glucose in normal and growth hormone transgenic mice. Reduced glucose tolerance and the resulting elevation in blood glucose levels are evident in normal aging. Risk factors including sedentary lifestyles, excess weight, poor diet habits and hereditary factors exacerbated by aging may lead to hyperglycemia and diabetes. Pathological complications and mortality risks attributed to hyperglycemia are discussed in this chapter. Reduction of blood glucose by our DSP has strong implications for treatment and prevention of diabetes and alleviation of associated conditions.

Increased longevity is associated with reduced blood glucose. Systemic and cellular regulatory systems including pathways shared by growth (aging) and stress resistance (anti-aging) are robustly modulated in accordance with glycemic status. These pathways integrate to arousal, foraging and other active behaviors as well as sleep. Food consumption and sleep duration (along with duration of other behaviors from 24h time budget studies) is documented and discussed with respect to aging and impacts of the DSP.

Hyperglycemia is attributed to insulin resistance, but insulin deficiency is typically contributing. Our DSP was designed to enhance insulin sensitivity considered a key aspect for successful anti-aging interventions. While insulin resistance was not specifically assessed, lowered blood glucose reliably suggests impact of supplementation on insulin action. Establishing effects of DSP on insulin sensitivity is important for consolidating mechanisms of action. From a solution oriented perspective, however, confirming the associated impacts on glycemic levels is ultimately of greater significance.

Physical exercise offsets hyperglycemia. At the same time, poor glycemic control can diminish physical performance and exercise tolerance. Duration of daily spontaneous exercise was assessed in aging supplemented and control mice. Additional (indirect) benefits of supplementation may be attributed to upregulation of physical exercise.

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CHAPTER 5

TITLE

Reduced Blood Glucose, Enhanced Exercise and Shorter Sleep in Normal and Accelerated-Aging Mice: Effects of a Complex Dietary Supplement

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5.1 ABBREVIATIONS

AMPK Adenosine monophosphate-activated protein kinase

PPAR\(\gamma\) Peroxisome proliferator-activated receptor gamma

IGF-1 Insulin-like growth factor 1

(All other abbreviations are explained in text)

5.2 INTRODUCTION

Type 2 diabetes mellitus is a metabolic disorder marked by elevated blood glucose. Prevalence of diabetes is on the rise with nearly two million new cases diagnosed each year in the US among adults [1]. The underlying causes for type 2 diabetes are insulin resistance and insulin deficiency, but the relative contribution of each mechanism can vary between individuals [2].

According to the most recent reports from the American Diabetes Association [1] approximately 26 million adults have diabetes but another 79 million (i.e. one quarter of the US population) suffer from prediabetic hyperglycemia. Hyperglycemia can result in a number of pathological conditions that greatly increase morbidity and mortality risks.
Cardiovascular mortality and stroke are 2–4 times higher in diabetics. Heart disease and stroke were implicated in over 80% of deaths among diabetic patients over the age of 65 years. About 60–70% have some form of neuropathy, and an equal percentage have high blood pressure. Diabetes is also the leading cause of kidney disease, and diabetic retinopathy is a major cause of blindness in persons aged between 20–70 years. Persons suffering from diabetes are 29 times more likely to be blind [3].

Diabetes, and milder forms of hyperglycemia, are associated with aging [1]. Risk of developing diabetes nearly triples in persons aged over 65 years [1]. Poor glucose tolerance, reduced glucose metabolism and elevated blood glucose are reliable indicators of aging [4–6]. Exceptionally long-lived mutant mice have low blood glucose levels [7,8]. Reduced blood glucose is also a ubiquitous phenotype of caloric restriction [9,10], the most reliable intervention for increasing lifespan of animals [6,11]. Aging rates reflect energy balance, and glycemic levels can affect the status of energy sensing pathways [6]. In this fashion, careful glycemic control is not only critical for diminishing risk of clinical pathologies, but is directly linked to aging rates as well.

Insulin is the main glucoregulatory hormone [12–14]. It controls rates of glucose uptake and utilization and has further impacts on lipid and protein metabolism [13]. Insulin resistance and insulin deficiency are the hallmarks of diabetes [1,2]. However, insulin resistance per se does not constitute diabetes, provided that resistance can be counterbalanced by higher insulin production. Likewise, mild insulin deficiency can be tolerated if peripheral cells retain adequate sensitivity. It is important to emphasize that ultimately, the pathological and metabolic deficits are attributed to glucose levels, reflecting imbalance between cytoplasmic and blood glucose, as opposed to levels of insulin production or resistance per se. Having said that, diabetic patients almost always exhibit both poor insulin sensitivity and deficient insulin production [2]. This means that counter-compensatory action is not possible.

This work describes the effects of a complex dietary supplement (DSP) on fasting and post meal glucose levels in aging and old mice. Diabetes is considered a condition promoting aging and is strongly associated with increased oxidative conditions. Our DSP
was formulated to target five mechanisms of aging including oxidative/nytrosative stress, inflammatory processes, membrane integrity, mitochondrial dysfunction and insulin resistance (glucose intolerance). We showed that DSP treatment reduced markers of oxidative and nytrosative damage, boosted mitochondrial function [15–17] and reduced symptoms of inflammation (unpublished) in aging mice. In addition, youthful cognitive and motor functionality was rescued in old age [15,16,18] and an 11–28% longevity extension was achieved [19]. Currently we report that fasting and post-meal glucose levels were reduced in supplemented mice (which were not attributed to altered food consumption). Spontaneous exercise duration was elevated while duration of rests between bouts of activity was reduced. These findings are indicative of better energy balance and enhanced glucose metabolism. The role of glycemic control in modulation of aging rates is in line with longer lifespan and otherwise more youthful phenotypes of old supplemented mice [15–18,20; Ch. 3,4,6].

Given the numerous complications of hyperglycemia [1,14,21–30], our DSP is expected to yield considerable health benefits provided that impacts translate to humans. In addition to potentially diminishing risk of developing diabetes our DSP may also reduce onset of several hyperglycemia-induced complications which may already be in progress before hyperglycemia is diagnosed.

Here we address the mechanisms of hyperglycemia-induced pathogenesis. The clinical and gerontological consequences of altered glucose metabolism are presented and discussed with respect to dietary supplementation. We also discuss mechanisms contributing to metabolic shortfalls in cardiac and skeletal muscle with implications for exercise performance in hyperglycemic animals. Based on present and previous findings, we identify possible cellular and endocrine pathways that may be involved in mediating the biological actions of our DSP. We also show that the DSP unexpectedly resulted in reduced sleep. The relevance of this finding is addressed with respect to endocrine regulatory systems.
5.3 METHODS

Animals: Test animals were normal C57BL/6 mice (Nr) and growth hormone transgenic C57BL/6×SJL hybrid mice (Tg). Tg mice have shorter lifespan, double the normal body size and show rapid motor and cognitive aging, altered energy metabolism and modified behavioral time budgets [see: 15,18,31–33]. Husbandry met all Canadian Council for Animal Care standards. Mice were housed in standard plastic cages with rodent chow (Teklad® 8640) and water provided ad libitum (room temperature: 23±2ºC; 12/12 hr light/dark photoperiod).

Complex dietary supplementation: We designed a complex dietary supplement (DSP) formulated to target five mechanisms associated with aging: oxidative stress, mitochondrial function, membrane integrity, inflammation and insulin resistance. Composition of the DSP, biological effects of components, dosages and detailed preparation protocols are found in [15,20]. The DSP was administered daily in home cages soaked onto a small square bit of bagel, as previously described [16,20].

Sugar-rich diet: A subset of Tg mice were provided with ad libitum access to both rodent chow and sugar cubes in separate compartments on their cage hoppers (starting at three months of age and maintained for life). Prior to three months, these mice were fed only rodent chow. Experimenters had no control over the amounts of either food consumed, although earlier work showed that Tg mice prefer sucrose more than do Nr controls and they maintain this preference for life [33]. Unless otherwise specified, all other mice used in experiments were maintained on ad libitum standard rodent chow for life (Teklad® 8640).

Behavioral time budgets: Full behavioral time budgets were composed for Nr and Tg untreated and supplemented males (n=40) over 24 hours (ages: 4–26 months). Mice were acclimated to the time budget arena for two days and video recording took place on the third day (apparatus described in [31]; see photograph in Appendix: Fig. A1). Video recordings were reviewed and the duration of behavioral activities was scored at one second intervals. All behaviors were categorized as: general locomotion, rest (immobility), sleep, grooming, nest building, drinking, eating, running on exercise wheel
and climbing on the wire mesh ceiling. General locomotion results were previously reported [15]. Here we report effects of genotype and the DSP on feeding, drinking, sleeping, rest, grooming and exercise duration. Exercise duration was calculated by adding time spent running on the exercise wheel and climbing on the wire mesh ceiling.

**Food consumption:** Mice were singly placed in a standard cage with wire mesh floor to prevent coprophagy. Pellets of rodent chow (12–14 pieces) were glued to the bottom of a small Petri dish (with non-toxic silicone) and placed in the cage (see: Fig. A2 in Appendix). Trials ran for five days or until most of the food was eaten. Cages were monitored twice daily to ensure apparatus integrity. The weight of the dish before and after the trial was recorded and divided by the duration of the trial to obtain rate of food consumption. To account for body size differences consumption rate is reported as a function of body mass (units: mg food/g body mass/day).

Mice fed *ad libitum* with a choice of sugar had separate dishes of chow and sugar cubes. Experimental protocol and calculation of food consumption rate were otherwise unchanged. Water, room temperature and light cycle were identical to the main mouse colony.

**Blood glucose:** A small drop of blood was obtained by tail vein prick with a 25G needle (mid-light photoperiod). Blood glucose levels were measured with an Accu-Chek® Compact Plus Blood glucose monitoring system. All mice tested were awake and mobile for at least 20 minutes prior to testing. Fasting glucose levels were collected following six hours of food deprivation. After, mice were given access to food for 10 minutes and post-meal glucose was measured 30 minutes following the feeding session. Mice on sugar-rich diets were given both sugar and rodent chow; mice on standard diets were given chow only. The amount of food consumed in the 10 minute inter-trial period was recorded.

*Average daytime glucose levels:* (In mice on sugar-rich diets only). Non-fasting daytime glucose levels were collected at randomly selected times during the light photoperiod on two separate occasions (~2 weeks apart). This measure of blood glucose
was considered to roughly approximate mean glucose levels during normal daily metabolic activity of mice.

**Statistical analyses:** We analyzed data using ANCOVA (covariate=age). If significant, age was included as a covariate; otherwise ANOVA was used to differentiate between genotype, treatment and gender. Where effects of gender were not significant, sexes were pooled. A t-test was applied for data represented by single independent variables. All age-related regressions were approximated with a linear fit. Analyses were performed using Statistica® 6.0 software.

### 5.4 RESULTS

**Blood glucose:** Levels of blood glucose were measured in mice during the light photoperiod following 6 hours fasting and again 30 minutes following a meal (standard rodent chow). Mice were between 4 and 26 months of age but effects of age were not resolved ($r^2=0.016$); (Nr n=26; Tg n=13). A significant effect of body mass was resolved for fasting glucose (ANCOVA, covariate=mass, main effect: $p=0.002$) and post-meal glucose ($p=0.050$). Fasting glucose was positively correlated with body mass in pooled genotypes ($r^2=0.207$; $p=0.003$) and in Nr mice ($r^2=0.204$; $p=0.018$). Post-meal glucose was also positively related to body mass but correlations were weaker. Since Tg mice are nearly twice the size of Nr animals, testing for the effects of mass and genotype in the same analysis would introduce a confound. Therefore, effects of DSP treatment were evaluated separately for Nr and Tg mice using ANCOVA (covariate=mass).

Both fasting and post-meal blood glucose was reduced in supplemented Nr mice by 24% and 17%, respectively, compared to untreated controls ($p=0.030$; $p=0.038$; Fig. 1A). Supplemented Tg mice showed a 13% reduction in fasting glucose ($p=0.050$) and a non-significant 10% reduction in post-meal glucose, compared to respective untreated controls (Fig. 1A).

To check whether the difference in post-meal glucose could be due to the amount of food consumed prior to measurement, we recorded the weight of chow eaten by each mouse (Fig. 1B). No differences in meal size were observed between treatments in either
genotype (Fig. 1B), confirming that effects on blood glucose were independent of amounts of food ingested.

Effects of DSP treatment on glucose levels were also tested in mice on a sugar-rich diet \(^{23}\) (Tg mice only; both genders; ages: 6–10 months, no effect of age; n=22). Meal sizes prior to recording post-meal glucose were similar between all groups (data not shown). Three measures of blood glucose levels were collected (see: Methods): (a) average daytime, (b) fasting, and (c) post-meal glucose. Average daytime glucose was assessed twice (~2 weeks apart) without fasting during the light photoperiod. No differences in average daytime glucose levels were seen between treatments in Tg females. However, supplemented Tg males showed a 22% reduction in average blood glucose compared to control Tg males (Fig. 2). Untreated female Tg had 17% lower blood glucose compared to untreated Tg males, but nearly identical to that of supplemented Tg males (Fig. 2). On measures of fasting and post-meal blood glucose no effects of sex or DSP treatment were observed (Fig. 2). Overall, post-meal glucose was significantly higher compared to fasting levels (p<0.0001), but only marginally higher compared to average daytime glucose measures.

**Food and water consumption:** When computing daily time budgets of mice we also scored duration of feeding and drinking over 24 hours (Table 1). Drinking duration was nearly identical for all mice which may indirectly reflect fluid consumption rates. Likewise, no impacts of genotype or treatment were resolved for duration of feeding; however, some variability was evident (see: Table 1). Nr supplemented mice spent 20% less time foraging than Nr controls, but strong significance was not attained (p=0.137). However, when only old (>1.5 years) Nr mice were tested, a significant 44% reduction in feeding duration was significantly resolved with ANOVA. Effects of age were otherwise non-significant for both behaviors.

To assess actual food intake we quantified the amount of chow consumed over a five day period (this was done at a later date and in a different subset of mice; ages 9–18 months). Figure 3A illustrates the rate of food consumption per day as a function of body

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\(^{23}\) Sugar-rich diet explained in *Methods*
mass. For Nr mice (n=36), no differences in food consumption rates were resolved between genders or treatments with ANOVA. Mass specific food consumption rate for Tg male mice (n=9) was 52% lower compared to Nr males (p<0.01; Fig. 3A). While gross food consumption was nearly identical (data not shown), because Tg mice are roughly twice the size of normal animals, mass specific consumption rates were significantly different. DSP treatment did not influence food intake in Tg males or females (Fig. 3A).

Sugar preference: Food preference between sugar and standard rodent chow (Teklad® 8640) was evaluated in Tg mice (Fig. 3B). Mice were provided with both standard mouse chow and sugar cubes ad libitum from three months of age. Food intake was monitored over a two week period at ages of 8–16 months (no age effects; n=18). Amount of total food consumed was not affected by DSP treatment (data not shown).

The percentage of sugar in total food consumed was 48% and 41% for untreated and supplemented females, respectively; and 43% and 63% for untreated and supplemented males, respectively (Fig. 5B). ANOVA (post-hoc SNK) resolved a significant effect of DSP treatment in males only (p=0.015). This difference amounted to a 46% increase in sugar preference. Supplemented males also had a higher preference for sugar than any of the females (ANOVA: p=0.032; Fig. 5B).

Spontaneous daily exercise: Duration of spontaneous exercise was assessed in a time budget arena over 24 hours in male mice (n=40). Mice exercised on a running wheel and by climbing a wire mesh ceiling (14 cm above floor), both of which are energetically demanding.

Tg mice: Exercise duration of untreated Tg mice was consistently low (0.2 hours/day) across all ages (Fig. 4). Supplemented Tg exercised more when young (1.2 hours/day) but duration diminished with age (Fig. 4). Exercise duration in old (>10 months) supplemented Tg was equivalent to untreated counterparts (Fig. 4). Slopes of linear regressions for exercise duration with respect to age were significantly differentiated (homogeneity-of-slopes ANCOVA: p<0.04) reflecting that the DSP had a significant impact on exercise only in younger Tg mice.
**Nr mice:** Duration of spontaneous exercise in Nr greatly exceeded that of Tg animals across all ages (Fig. 4). Both supplemented and untreated Nr showed significant age-related declines in exercise duration; however supplemented mice exercised more at all ages (Fig. 4). Mean exercise duration was 73% higher in supplemented Nr mice (1.9 hours/day) compared to untreated controls (1.1 hours/day). This difference was significantly resolved with ANCOVA (covariate=age; p<0.05; Fig. 4). Unlike Tg mice, higher exercise duration in supplemented Nr animals extended into older ages (Fig. 4).

**Duration of daily rest:** Duration of immobility (rest) was recorded in supplemented and untreated Nr and Tg males (n=40) over 24 hours in time budget arenas. A mouse was considered resting when it remained immobile for longer than one second but was not asleep. Generally mice rested in short (1–10sec) intervals separated by longer bouts of locomotion, exercise, foraging, grooming or other active behaviors. Duration of total daily rest was not resolved between genotypes; however, Tg mice showed greater individual variability compared to Nr animals (compare Figs. 5A and B).

**Nr mice:** Rest duration in Nr mice was virtually identical for both treatments when young (~4 months old) (Fig. 5A). Untreated Nr mice showed a significant elevation in rest duration with age (linear regression: p<0.05) resulting in a 90% increase by 2 years of age (Fig. 5A). Conversely, supplemented Nr showed no change in resting duration with age, effectively maintaining youthful (low) levels of resting even when 2 years old (Fig. 5A). At 2 years of age a 55% difference was observed between treatments and this was resolved with ANCOVA (covariate=age; p<0.03; Fig. 5A).

**Tg mice:** Duration of rest in Tg mice did not show age-related correlations in either treatment (Fig. 5B). A 37% increase in mean resting duration in supplemented mice was not statistically resolved (Fig. 5B).

**Daily sleep duration:** Duration of sleep was recorded in a time budget arena over 24 hours. Age ranges were 4–26 months for Nr (n=18), and 4–15 months for Tg mice (n=22). Weak positive age-related trends were observed in Tg mice ($r^2=0.058$; p=0.279) but no correlation was seen in Nr ($r^2<0.001$; p=0.945). Homogeneity-of-slopes ANCOVA
(covariate=age) found no significant difference between slopes of sleep duration with age (p=0.357) so ages were pooled.

Nr supplemented mice slept 2.5 hours less than untreated controls (19% reduction; ANOVA: p<0.01; Fig. 6). Similarly, supplemented Tg mice slept 1.7 hours less compared to untreated Tg (12% reduction; ANOVA: p<0.05; Fig. 6). An overall effect of genotype was also resolved: sleep duration of Tg was 14% greater compared to Nr mice (ANOVA, combined treatments; p<0.0001). This difference is smaller than the previously reported 26% increased Tg sleep [31], which may be explained by modifier selection [see: 34].

**Grooming and paper shredding:** Due to the setup, grooming and paper shredding (nest building) behaviors were difficult to discriminate. To avoid arbitrary scoring, both behaviors were combined into a single measure. In fact, grooming and paper shredding in rodents are very closely related falling under the same regulatory control [35]. Increased grooming in rats was paralleled by similar increases in paper shredding [35].

Duration of grooming/paper shredding was not correlated with age in Nr mice, but a strong main effect of diet was observed (Fig. 7A). Supplemented Nr spent 52% more time grooming/shredding compared to untreated controls across ages (ANOVA: p<0.006). Grooming and shredding in untreated Tg mice declined by over 50% over their lifetime (r²=0.43; p=0.027) while supplemented Tg showed a mild age-related elevation in grooming/shredding duration. At ages of ~15 months, grooming/shredding duration was 80% greater in supplemented Tg mice. Homogeneity-of-slopes ANCOVA (covariate=age) resolved a significant difference in age-related slopes between treatments (p<0.05).

### 5.5 DISCUSSION

On standard rodent chow, treatment with our DSP resulted in significantly reduced fasting and post-meal blood glucose levels in Nr mice. Similar but lesser effects were observed in Tg (Fig. 1A). On a sugar-rich diet, fasting and post-meal glucose levels were not affected by supplementation (Fig. 2). However, it is important to note that sugar-rich
diets were only studied in Tg mice, who had poor responses to DSP treatment even on standard chow. Hence, impacts of DSP on blood glucose of sugar-fed Nr mice may not be excluded.

Average blood glucose levels are best reflected by glycosylated hemoglobin (HbA$_{1C}$) concentrations, however limitations to this method exist [see: 36]. Moreover, information obtained from direct glucose monitoring and the HbA$_{1C}$ test are fundamentally different [36]. Levels of HbA$_{1C}$ were not assessed at the time of study. To roughly approximate daytime average blood glucose levels (in sugar-fed Tg mice), animals were sampled on two random occasions during the light photoperiod. Periodically sampled blood glucose shows a strong correlation with HbA$_{1C}$ [37] indicating that repeated random glucose sampling may accurately reflect average blood glucose.

Despite no effects of the DSP on fasting and post-meal measures in Tg mice, average daytime blood glucose was reduced by 22% in supplemented males (Fig. 2). The relatively large sample size (n=22), repeated measures, and high statistical significance (p=0.012) suggest that random errors were unlikely to introduce bias. It is possible that on top of systemic (hormonal) regulation of blood glucose levels, mice may also employ behavioral regulatory control; perhaps through regulating meal size or better balancing food intake with energy expenditure. For instance, Tg mice fed sugar greatly increased their activity levels and slept less [33].

Feeding studies confirmed that daily food consumption was not affected by DSP treatment on either standard chow or sugar-rich diet (Fig. 3A). Body mass was also unaffected by treatment (data not shown). Tg mice on the sugar-rich feeding regime had free choice of rodent chow or sugar cubes ad libitum. It is interesting that sugar preference of supplemented Tg males (the only group with reduced blood glucose) was the highest among all mice, by about 46% (p=0.019). This suggests that DSP treatment allowed for better glucose management despite higher sugar intake. These effects, however, did not occur in Tg females.
Overall, blood glucose levels in our animals (on standard diets) were comparable to normal glucose levels reported for C57BL/6 mice by others\(^{24}\) [4]. Remarkably, we did not observe strong age-related correlations, although a weak positive association was detected. Therefore, our mice may not be strongly subject to age-related hyperglycemia. Despite little room for improvement, we observed significant impacts of the DSP on average, fasting and post-meal glucose. This indicates that glucose tolerance was substantially enhanced suggesting possible therapeutic applications for preventing or lowering the risk of developing metabolic disorders such as type 2 diabetes in humans.

**Glucose intolerance and hyperglycemia:** Glucose tolerance may decrease with age [5,6] resulting in metabolic consequences and elevated blood glucose levels [4]. In healthy persons, fasting and post-meal glucose levels were higher in individuals aged 55+ years compared to 24 year old adults [38]. Therefore, hyperglycemia and diminished glucose tolerance is not strictly an attribute of age-related complications, but is evident in ‘healthy’ aging as well.

Hyperglycemia is considered pathological if either fasting or post-sugar load plasma concentrations of glucose exceed normal ranges [12]. Persons with particularly high glucose levels may be diagnosed with diabetes mellitus [12,39]. The generally accepted criteria for differentiating between normal glycemic levels, hyperglycemia and diabetes are based on WHO recommendations\(^{25}\) [12]. Measure of HbA\(_{1c}\) can also be used in conjunction with glucose sampling [12].

Type 1 (insulin-dependent) diabetes comprises only 10% of diabetes cases worldwide, while 90% are attributed to type 2 [40]. Type 1 diabetes is an autoimmune condition resulting in destruction of pancreatic β-cells [39]. Depending on rate of β-cell loss, patients may develop either absolute or partial insulin deficiency. Save for mild cases, individuals become dependent on exogenous insulin for survival [39]. The etiology of type 2 diabetes is different from type 1, being attributed to insulin resistance and lack of compensatory insulin secretion [39]. Further reference to diabetes here means type 2.

\(^{24}\) Some studies report blood glucose in mg/dl. We measured glucose in units of mmol/L. Comparisons can be made using a conversion factor: 1 mmol/L = 18.0182 mg/dl

\(^{25}\) Hyperglycemia disorders: fasting glucose < 7.0 mmol/L and/or 2 hours post-glucose load < 7.8 mmol/L. Diabetes: post-glucose load levels of ≤ 11.1 mmol/L. (Glucose load = 75g glucose orally).
Older individuals are at higher risk of developing diabetes [41,42], however other factors such as obesity [2,5,12,43], genetics [44], sedentary lifestyles [12] and poor diet [24] comprise greater risk factors. The underlying cause of glucose intolerance and hyperglycemia in diabetes is primarily resistance to insulin [39,45,46]. However, insulin deficits commonly accompany insulin resistance, resulting in poor compensation [2,39].

Insulin is the chief glucoregulatory hormone [12–14] effectively controlling total-body glucose metabolism [13]. When glucose levels rise, such as after a meal, high insulin concentrations are required to drive glucose utilization [13]. Elevated insulin also suppresses glucagon [47], thereby restricting hepatic gluconeogenesis [48]. This leads to clearing of glucose from blood and eventual return to normal fasting levels. In the fasting state, insulin regulates hepatic glucose production maintaining steady plasma glucose concentrations [13]. In healthy individuals, this regulatory system is very precise, and even minor shifts in insulin concentrations strongly alter glucose metabolism [13].

Ultimately, regulation of glucose metabolism depends on insulin receptors on peripheral cells. Earlier studies observed lower numbers of functional receptors in insulin resistant humans [49] and mice [50]. Normally, glucose utilization achieves maximal flux even at <50% receptor occupancy, suggesting that cells express “spare” receptors [13]. Therefore, considerable receptor loss (or binding affinity) may be tolerated without large metabolic consequences. Decreased insulin receptors are usually seen in mild glucose intolerance disorders [51]. Advanced insulin resistance and hyperglycemia also involve post-receptor signaling dysfunctions [49,51].

Elevated insulin production by β-cells can offset peripheral resistance to insulin. However, diabetic patients are unable to sufficiently compensate for insulin resistance; either because insulin resistance is too great, or because sufficient insulin production by β-cells is not possible [2,39]. Thus, both insulin resistance and insulin deficiency contribute to diabetes [2]. Defects in insulin action per se, are not inherently lethal, providing that blood glucose levels and cytoplasmic concentrations can be managed within normal margins. The underlying causes of death in diabetic patients are cardiovascular disease (52% cases), cancer (14% cases) and renal disease (11% cases).
Only 3% of diabetic patients were diagnosed with diabetes as the primary cause of death [52]. Health hazards and mortality risks are secondarily exacerbated by hyperglycemia and defective cellular metabolism.

Chronic hyperglycemia is closely linked to damage and failure of several organs including the heart, blood vessels, kidneys, eyes and nerves [3,14,21,23,24,29,39]. Conditions that account for most of the pathology associated with hyperglycemia largely trace to microvascular complications [29,30]. These commonly include neuropathy, nephropathy and retinopathy [21,22,27–30]. Apoptosis of vascular tissue cells induced by hyperglycemia [53,54] appears to be the principle mechanism in microvascular conditions and particularly in retinopathy [21]. Retinopathy is especially difficult to reverse once symptoms manifest [22,55]. Additional cardiovascular complications can be attributed to hypertension, as high plasma glucose interferes with nitric oxide-induced vasodilatation [25,29]. Glomerular hypertension is thought to be an additional cause of injury in kidney disease [27]. Hyperglycemia is also associated with increased risk of atherosclerosis [26,56,57] and heart failure [4,23,24,26], however mechanisms of the latter are not fully understood. While effects of hyperglycemia are usually studied in diabetic patients, high post-meal blood glucose can independently predict risk of cardiovascular disease [56]. Likewise, elevated HbA1C levels independently predict risk of heart, eye, kidney and nerve disease [23].

Hyperglycemia may develop well in advance of obvious symptoms; hence cellular damage may go unchecked for years. Glucose and insulin resistance can be present for more than a decade before diagnosis [46]. A large number of patients diagnosed with diabetes already display microvascular complications which may be difficult to arrest or reverse even with intense glycemic control, however some remission is possible [22]. Insulin therapy [58] and treatment with drugs (such as Metformin) [59] can help regulate blood glucose levels. However, this does not rescue insulin sensitivity. Better diet, physical exercise and weight-watching are still the best treatment options [50,60,125].

Molecular mechanisms underlying insulin resistance, poor β-cell compensation, and general glucose intolerance are complex and vary among subjects [39]. Specific
etiologies are poorly understood [39]. A variety of systemic and cellular targets are known to contribute to both insulin deficiency and insulin resistance. Reactive oxygen species (ROS) and inflammatory cytokines are ultimately involved [12,14,23,24,41,45,61,62]. Experimental evidence showed that low-grade prolonged ROS stress (more similar to aging than acute events) directly induced insulin resistance in cells [63]. Exposure to hyperglycemia, both *in vivo* and *in vitro*, induced ROS production [64]. In this fashion, hyperglycemia can induce further insulin resistance and the entire system can enter a state of dysregulatory self-perpetuation if left unchecked. In addition, mitochondrial dysfunction was implicated in post-receptor insulin resistance [65]. Decreased mitochondrial activity can result in accumulation of fatty acyl CoAs and diacylglycerol in the cytoplasm which can deactivate insulin signaling [65].

Insulin resistance was one of the age-related processes specifically targeted by our DSP. Other targets included oxidation/nitration, inflammation and mitochondrial function. Eleven components in our DSP enhance insulin sensitivity [27]. However, given the involvement of ROS, inflammatory cytokines and mitochondrial dysfunction in insulin resistance, it follows that virtually all of the ingredients may be beneficial. We reported that supplementation significantly reduced oxidative and nytrosative damage [15,17] and markers of inflammation (unpublished), and boosted mitochondrial activity in aging mice [15,20]. This suggests that insulin sensitivity may also be rescued. Indeed, the present findings are consistent with enhanced insulin sensitivity.

Insulin resistance was not actually measured here; however, insulin action is tightly correlated with glycemic levels [66]. Hence, reduced blood glucose in supplemented mice is likely indicative of augmented insulin sensitivity. Irrespective of whether insulin sensitivity was altered, perhaps of greater overall importance is the finding pertaining to glycemic control. As described above, it is ultimately not insulin resistance per se, but rather high plasma glucose that is responsible for mediating the array of pathologies implicated in diabetes and hyperglycemic disorders. Our DSP impacted both fasting and post-meal blood glucose in healthy mice indicating that general thresholds for glucose tolerance were lowered. Assuming that effects translate to humans, treatment may
considerably reduce the risk of developing hyperglycemia along with all associated pathologies.

Glycemic control in healthy individuals is of increasing importance given that 'silent' hyperglycemia may inflict considerable and partially irreversible damage well in advance of symptom onset [46]. For example, poor post-meal glucose clearance, a major risk factor for cardiovascular disease [56], is not accurately determined by HbA₅₅C tests and fasting glucose measures. Self-glucose monitoring may also be inaccurate as meal size and additional factors can interfere. Therefore, unless meticulous glucose monitoring regimes become a norm, preventative measures remain the only effective intervention. Active lifestyle and balanced diet are of principal importance; however, additional sizable benefits appear possible through complex dietary supplementation.

**Hyperglycemia and glucose metabolism:** Glucose metabolism in humans was found to decline by up to 50% between 20 and 65 years of age, with further declines in more advanced ages [4]. In healthy individuals, metabolic decline may reflect somewhat lesser demands for energy with age; however, age-related glucose intolerance [5,6] is almost certainly implicated. Poor glucose metabolism in diabetics is evidential to that effect [51]. Additionally, factors such as declining mitochondrial function may further contribute [65].

Altered glucose metabolism was implicated in pathogenesis of diabetic neuropathy (of the peripheral nervous system) [67], cardiac disease and skeletal muscle dysfunction [68], among other effects [69]. In addition to glucose utilization, insulin regulates protein and lipid metabolism [69]. Pathological consequences with respect to the entire spectrum of insulin-mediated metabolic control are reviewed [69]. However, the present discussion of metabolic deficits is restricted to those directly mediated by hyperglycemia. We will briefly address impacts of hyperglycemia on whole organism metabolic control as well as cellular metabolism in specific tissues (cardiac and skeletal muscle).

**General metabolism and the metabolic syndrome:** Hyperglycemia can results in weight gain through increased lipid storage [70]. High insulin levels were shown to
suppress adiponectin\textsuperscript{26} \cite{71}, a fat-derived hormone involved in regulation of glucose and fatty acid metabolism \cite{70}. Adiponectin upregulates AMPK \cite{72} which helps induce glucose uptake in muscle \cite{72,73} resulting in elevated mitochondrial energy production. Adiponectin can also suppress glucose production in the liver \cite{74}, meaning that low levels of adiponectin will disinhibit gluconeogenesis.

Insulin becomes elevated in response to high blood glucose \cite{13,66}. In this way, hyperglycemia causes reduced metabolism by mediating insulin-dependent suppression of adiponectin. This results in diminished energy production by cells (i.e. energy shortage) and slow lipid oxidation in adipocytes, leading to fat storage, weight gain, and onset of the metabolic syndrome associated with diabetes and poor glucose tolerance. Reducing blood glucose will shift this system to reverse \cite{6} leading to higher metabolisms and further reduction of blood glucose \cite{6}. Leptin, another hormone produced by white adipose tissues \cite{75} involved in regulating energy homeostasis \cite{76} may also be implicated to some extent [see: 6].

**Cardiac Muscle:** In cardiomyocytes of healthy adults, carbohydrates and free fatty acids provide the metabolic fuel for ATP synthesis \cite{77,78}. In hyperglycemic persons, carbohydrate metabolism is diminished which is compensated by higher oxidation of fatty acids \cite{78}. Fatty acid metabolism increases oxygen requirement per molecule of ATP produced, compared to glycolytic ATP synthesis \cite{79}. In addition, higher availability of fatty acids elevates expression of uncoupling proteins in mitochondria \cite{80}. In this fashion, the shift towards greater fatty acid metabolism leads to an increased oxygen requirement \cite{68,78}. Oxygen starvation is expected at lower cardiac output levels. Disproportionately high fatty acid oxidation was indeed correlated with decreased contractile work \cite{81}. Hyperglycemia also promotes atherosclerosis \cite{57} which further diminishes myocardial blood supply \cite{82} raising incidence of silent ischemia \cite{83}.

In insulin resistant animals, hyperglycemia increases glucose uptake \cite{84}, which may be expected considering the steeper concentration gradient. Glucose delivery is nonetheless reduced due to insulin resistance \cite{78}, but hyperglycemia per se appears to be

\textsuperscript{26} Adiponectin is also referred to as Acrp30 in literature.
partly offsetting [84]. At the same time, following a short hyperglycemic challenge in non insulin resistant persons, glucose uptake rates were significantly diminished up to 24 hours following the hyperglycemic event [85]. Again, this was specifically attributed to hyperglycemia per se [85]. Thus, transient elevation of blood glucose can cause long lasting inhibition of glucose delivery thereafter. Given the metabolic consequences of carbohydrate substrate shortage in cardiomyocytes [78], it becomes apparent why poor post-meal glycemic control in particular, strongly predicts risk of heart disease [56]. Likewise, this also explains the lack of correlation between HbA1c levels and impaired cardiac metabolism [68].

**Skeletal muscle:** Contrary to the heart, hyperglycemia-induced metabolic deficits in skeletal muscle do not reflect carbohydrate substrate shortage. Muscle energetics and phosphate metabolism were normal in diabetic patients at rest [68]. During exercise, glycogen levels and substrate availability were not limiting [68]. However, oxygenation, and post-exercise reoxygenation were significantly slower in diabetics, implying poor exercise tolerance [68]. This is likely explained by impaired oxygen delivery to muscle due to hyperglycemia-induced microvascular complications [29,30]. Indeed, elevated levels of HbA1c are associated with reduced exercise capacity [68]. This presents an additional challenge for persons with diabetes, as physical exercise is instrumental in glycemic control, but at the same time, it is poorly tolerated both on the cardiac and skeletal muscle levels.

**Exercise and glycemic control:** Daily exercise duration in mice was presently assessed from 24 hour behavioral time budgets. Impacts of the DSP on exercise duration in Tg were poorly resolved except for younger ages (Fig. 4). However, supplemented Nr mice showed significantly higher duration of spontaneous exercise compared to untreated controls throughout lifetime by over 70%; albeit age-related declines persisted (Fig. 4). We previously reported that locomotion (non-intensive physical activity), was significantly upregulated in old DSP treated animals [15]. This was attributed to boosted mitochondrial function and reduced markers associated with general aging [15–17]. Presently, it appears that better glycemic control may be contributing.
Given that untreated mice did not exhibit age-related elevations in blood glucose, it is unlikely that hyperglycemia-induced microvascular damage in skeletal muscle was a major factor limiting exercise duration in our animals. Regardless, better glycemic control may play a partial role. Also, lower blood glucose, in spite of unchanged food consumption, suggests enhanced glucose metabolism. This implies that mitochondria in tissues limited by carbohydrate substrate availability (such as cardiac muscle) may run on a higher relative proportion of carbohydrate fuel. This requires less oxygen [79] and should permit greater cardiac output when needed to support intense exercise.

A more plausible relationship of reduced blood glucose and exercise duration is that higher levels of spontaneous exercise induced by supplementation may improve glucose tolerance. Indeed, physical exercise is negatively associated with risk of developing glucose intolerance and insulin resistance [60]. Thus, boosted physical activity may independently offset insulin resistance and glucose intolerance.

Some evidence suggests that exercise can increase adiponectin levels, which should increase insulin sensitivity and lower blood glucose [86]. However, others found no lasting effects of exercise on adiponectin [87]. It appears that only transient activation of adiponectin is possible with acute exercise [88].

**Rest and exercise tolerance:** In addition to higher spontaneous exercise, daily duration of rest periods was significantly impacted by DSP in Nr aging mice (Fig. 5). Old untreated animals rested more frequently between bouts of physical activity. Conversely, supplemented Nr showed no increase in rest duration across ages. Even in the oldest animals, levels of rest remained unchanged from those in youth. Shorter rest was not compensated by longer sleep; in fact, sleep time was also diminished in supplemented mice (Fig. 6). Firstly, that DSP treated mice slept and rested less, naturally implies that active behaviors were upregulated. This was emphasized above and in previous studies [15]. However, examining patterns of rest duration may be relevant to other aspects such as exercise tolerance.

During initial analysis of behavioral time budgets, we recorded the duration of each individual rest episode. Due to logistic feasibility this was later replaced by only scoring
the total (combined) duration of daily rest. Duration of individual rest periods was scored for two untreated and one supplemented Nr, and one Tg supplemented mouse. All mice rested in short (1–10 second) periods in between longer bouts of physical activity. Rest duration rarely exceeded 10 seconds and never exceeded 1 minute. This was also visually evident from reviewing all time budget recordings. Hence, regardless of genotype or treatment, duration of a single rest episode was unchanged. Thus, variability in total daily rest duration largely reflects frequency of rest bouts. As supplemented old mice exhibited shorter total rest, we can arguably conclude they took fewer breaks in between active behaviors. This may reflect better exercise tolerance, given that reduced glucose tolerance is correlated with intolerance of exercise [89].

**Hypoglycemia and aging:** Reduced glucose metabolism and elevated blood glucose are key indicators of aging [4–6]. Hyperglycemia was implicated in the fast aging of senescence-accelerated mice [90]. Our Tg mice, also a model of accelerated aging, were presently found to have higher blood glucose than Nr animals. Conversely, long-lived mice such as the Ames and Snell dwarves show reduced plasma glucose levels [7,8]. Caloric restriction (CR) is the best known intervention for extending lifespan across a range of species [11]. CR was shown to lower blood glucose in rhesus monkeys [9] and mice [6,10]. One theory holds that the entire anti-aging mechanism in CR stems directly from reduced plasma glucose [explained in: 6]. Interestingly, the degree of blood glucose reduction achieved by our DSP (~20%) is comparable to that attained through CR [9,10]. CR in Ames dwarves did not further lower blood glucose [8], suggesting that a lower limit was already reached and further reduction may be harmful.

Besides lowered blood glucose, long-lived dwarf mice also exhibit increased stress resistance, reduced oxidative damage [7], protection from neuronal damage [91], better cognitive function [8] and, of course, increased longevity [7,8,92,93]. Calorically restricted animals share these phenotypes with the long-lived dwarves [6], but also show augmented mitochondrial function [94] and lower incidence of cancer [95]. Treatment with our DSP also yielded all of the above phenotypes. We showed that supplemented mice were more resistant to radiation stress [20]; had less oxidative damage [15], boosted
mitochondrial function [15–17], higher neuronal populations in brain [Ch. 3,6], enhanced cognition [16,18; Ch. 6], reduced cancer risk [Ch. 7] and an 11% longevity extension in Nr animals [19]. The current blood glucose results are consistent with all previous findings related to slower aging rates.

The Ames and Snell dwarves have smaller body size [7,8], poor fecundity [7] and less physical vigor [92]. For animals in the field and humans alike, these qualities are undesirable. Remarkably, our DSP treatment elevated exercise (Fig. 4) and physical vigor [Ch. 4], increased female fecundity [Ch. 8] and had no negative impact on adult body size [Ch. 8]. These additional functional benefits are likely traded off against greater otherwise possible increases in longevity. We believe that for the aging human population, extension of functional aging greatly outweighs benefits of mere longevity extension.

**Blood glucose, metabolism and longevity; the sirtuin link:** Longevity extension in our mice [19] may be partly due to better glycemic control, but other mechanisms undoubtedly contribute [15–18; Ch. 3,8]. Blood glucose levels can affect aging by modulating metabolic pathways already discussed above. Another metabolic regulatory pathway implicated in aging involves sirtuins (silent information regulators). Mammals have seven sirtuin homologues (Sirt1–7). Most homologues exhibit deacetylase enzyme action [96] regulating gene expression via chromatin modification [97]. Sirtuins link numerous cell functions to cellular energy status [97] interacting with stress resistance, cell cycle, DNA repair, insulin secretion, thermogenesis and metabolic pathways [96] and immunity [97]. Sirtuins are implicated in longevity and mediation of CR responses [96].

Sirtuin-1 (Sirt1) was shown to repress PPARγ [96,98], a regulator of white adipose tissue (WAT) cell gene expression, including the mouse aP2 gene [99]. The product of the aP2 gene promotes fat storage; hence inhibition of aP2 results in fat utilization [6]. Animals are thought to use WAT status to infer energy availability, thus greater fat utilization may signal food shortage and reset the pace of aging [see: 6]. Indeed, this pathway is one possible route for the anti-aging effects attributed to Sirt1 activation [100] (although other mechanisms were suggested [6,101]). Physiological action of Sirt1
extends to regulation of blood glucose levels and insulin secretion [102]. Sirtuins induce greater insulin secretion from pancreatic β-cells in mice [103]. In yeast, Sirt2 was shown to increase mitochondrial function [104]. We reported that our DSP also boosted mitochondrial activity in old mice [15,16]. Thus, the present findings, as well as previously reported effects of our DSP, are consistent with upregulation of sirtuins.

Resveratrol is the best recognized dietary activator of Sirt1 [105,106]. Resveratrol is now included in the latest formulation of our DSP [Ch. 7] but was not part of the formula at time of present study. Ingredients included in our DSP, such as α-lipoic acid [107] and polyphenols (found in green tea) [108] also activate Sirt1. Other DSP components may also indirectly contribute. Hence, it is very likely that lower glucose levels, extended longevity, and additional phenotypes of supplemented mice may trace in part to upregulation of Sirt1. Assessment of Sirt1 activity, and other members of the sirtuin family, is an immediate future direction.

**Food consumption:** We already addressed the finding that average daily food intake was unaffected by DSP treatment on either standard rodent chow or a sugar-rich diet. Duration of feeding and drinking was also assessed in behavioral time budgets. Supplemented Nr mice spent 20% less time foraging (non-significant difference; Table 1). However, when only old (>1.5 years) Nr are compared, we find a significant 44% reduction in daily feeding duration in supplemented mice.

As amount of food consumed was not effected by treatment, it follows that older supplemented mice ingested more food per feeding session. We speculate that mice spent less time handling or ‘playing’ with food in favor of faster ingestion. There is some evidence to suggest that meal size can affect plasma glucose levels. Larger meals stimulated greater insulin secretion from β-cells in diabetic patients, and despite a higher sugar load, plasma glucose levels were actually lower in the 30–180 minute period following a large meal, compared to a smaller (half-size) meal [109]. This suggests that glucose may be better managed by intermittent ingestion of larger portions as opposed to more frequent intake of smaller-sized rations. It appears that the DSP induced a variation in foraging behavior, though differences were restricted to old mice. In any case, this
opens up the possibility for behavioral management of glycemic regulation independently of hormonal control.

Because food intake was unaltered by the DSP a general discussion of hormones regulating food intake and appetite is not included here. A detailed review can be found in [76]. However, we will address the biological action of one neuropeptide hormone – orexin (or hypocretin), as its regulatory role extends beyond feeding and food-seeking behavior [76] and may explain altered sleep in DSP treated mice.

**Sleep duration:** Sleep duration was found to be reduced in both Nr and Tg supplemented mice by significant margins (2.5–1.7 hrs/day; 19–12% reduction; Fig. 6). Some concern about curtailed sleep arose as functions such as memory consolidation [110] and stress resistance [111] are linked to sleep. However, DSP treated mice show both enhanced memory and learning [16; Ch. 6] and superior stress resistance [20]. The temporal organization of sleep/wake patterns, and further differentiation of sleep into early and late windows with opposite regulatory impacts [111], implies that overall sleep duration per se cannot accurately predict physiological and behavioral outcomes. In addition, there is an obvious fundamental difference between exogenous sleep deprivation (i.e. forcefully curtailed sleep) and endogenous adjustment of sleep patterns stemming from systemic regulatory systems.

A large number of endocrine agents affect sleep, and most also play a role in energy metabolism [for full review see: 112]. Orexins are of particular interest here. Activation of orexin neurons stimulates wakefulness and downregulates sleep, increases food consumption (appetite) and elevates locomotor activity [113]. The latter is needed to support food-seeking. Our DSP increased locomotor activity in mice [15] and reduced sleep (Fig. 6). These findings are consistent with orexin upregulation.

Food consumption in supplemented mice was unaffected (Fig. 3). It was shown that high levels of chronic orexin administration stimulated feeding, but there was no elevation in total food consumption over 24 hours [114,115]. Animals consumed more food in the light photoperiod balanced by reduced nighttime food intake [114]. We did not record daytime versus nighttime food consumption; however, it appears that
supplemented Nr mice ingested food faster when feeding (shorter feeding sessions) than untreated animals (see preceding discussion). This may be indicative of increased appetite. In this fashion, unchanged total daily food intake and presumably increased appetite are entirely characteristic of higher orexin levels.

Furthermore, orexins were found to increase grooming behavior in rodents [116]. Our behavioral time budgets showed that grooming was elevated in DSP treated Nr mice across all ages (Fig. 7). Longer grooming was also apparent in Tg mice, albeit only in old age.

Chronic orexin elevation does not alter body weight [115] and rodents with impaired orexin function do not exhibit energy imbalance [76]. Besides foraging/feeding, orexins stimulate arousal and suppress sleep [113,117]. Administration of orexins induced and maintained waking and general arousal [113,116], while knockdown of orexin reduced wakefulness and produced irregular sleep fragmentation [117]. Orexins are under tight circadian control [113], but can be independently activated in response to food deprivation [76]. In particular, hypoglycemia was shown to increase orexin mRNA expression [118,119]. This is expected, as glycemic levels are indicators of energy balance. Given that our supplemented mice had reduced fasting blood glucose we expect them to have higher orexin activity. This may explain shorter sleep duration in supplemented mice and correlates with augmented locomotor [15] and grooming behavior (Fig. 7).

An additional connection between glycemic levels and sleep duration may involve insulin. Insulin was shown to induce sleep in rats [120]. In addition, high insulin levels can also elevate circulating IGF-1 levels via inhibition of IGF-1 binding proteins [121]. IGF-1 was also found to facilitate sleep onset [122]. Lower glycemic levels in our supplemented mice suggest that insulin levels may also be diminished [66]. This may lead to additional downregulation of sleep [120].

**General considerations for laboratory animals:** It may be possible that our mice (and perhaps all laboratory rodents) chronically ‘oversleep’ compared to optimal sleep duration in the field. Since food is always provided *ad libitum* and in immediate vicinity,
food-seeking behaviors, otherwise crucial in the field, are virtually absent. Since mice never experience starvation or even mild food deprivation, the orexin system may be downregulated below normal levels causing longer sleep. In this fashion, the shorter sleep in our DSP treated mice may not reflect abnormal sleep reduction, but rather a return to optimal sleep duration. This would also explain the absence of any negative effects on sleep-associated functions in supplemented mice.

It follows that lab mice may overeat. Indeed, it was shown that laboratory mice ate roughly 20% more compared to wild mice when adjusted for weight [123]. Likewise, CR may have little impact on lifespan of wild mice [124], presumably because wild mice already reflect ‘caloric restriction’ compared to laboratory animals. Our DSP appears to obtain some benefits associated with CR without the need for reduced feeding. Alterations in orexins may contribute.

One manipulation may be to provide laboratory mice with food sources that are challenging to locate (i.e. mimic field conditions). In this fashion, animals will still ultimately consume food *ad libitum*, but the added food seeking requirement should activate the orexin system and associated benefits.

**Summary:** In this work we showed that treatment with a complex dietary supplement designed to ameliorate processes associated with aging significantly lowered fasting and post meal glucose in aging and old normal mice. This effect was not due to decreased food intake. Treatment also resulted in elevated levels of spontaneous exercise and reduced frequency of rest bouts between active behaviors. Supplemented animals showed reduced duration of daily sleep and increased grooming, suggestive of upregulated orexin activity. Collectively, these findings indicate that our dietary treatment improved energy balance and enhanced glucose tolerance. Human applications extend to better glycemic control and lower risk of developing hyperglycemic conditions such as type 2 diabetes. Hyperglycemia-associated complications such as cardiovascular disease, kidney failure, neuropathy, retinopathy, and exercise intolerance may also be offset.
5.6 ACKNOWLEDGMENTS

Authors extend special thanks to Aleksey Matviyenko for assistance with blood glucose measurements. Research funding supported by a grant to C.D. Rollo (NSERC).

5.7 FIGURES and TABLES

Table 1. Duration of feeding and drinking measured over 24h in a time budget arena for supplemented and untreated Nr and Tg males. No differences in duration or either activity were resolved between treatment groups. Ages: 3–27 months, effects of age not resolved

<table>
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<th>Genotype</th>
<th>Treatment</th>
<th>Feeding duration (h/24h)</th>
<th>Drinking duration (h/24h)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Unt</td>
<td>1.62h</td>
<td>0.18</td>
<td>9</td>
</tr>
<tr>
<td>Normal</td>
<td>Supp</td>
<td>1.30h</td>
<td>0.17</td>
<td>9</td>
</tr>
<tr>
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<td>0.16</td>
<td>11</td>
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<tr>
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<td>Supp</td>
<td>1.88h</td>
<td>0.16</td>
<td>11</td>
</tr>
</tbody>
</table>
Figure 1. Regular rodent chow: (A) Levels of blood glucose (mmol/L) in Nr (n=26) and Tg mice (n=13) (ages: 4–26 months) after 6 hr of fasting and 30 min following a meal. Fasting glucose was 24% (p=0.030) lower in supplemented Nr and 13% (p=0.050) lower in Tg mice compared to respective controls. Post-meal glucose was 17% lower in Nr mice compared to untreated Nr controls (p=0.038). Effects of treatment on post-meal glucose levels in Tg mice were not resolved (10%; p=0.565). A significant overall effect of genotype was found for fasting glucose: Tg were 17% higher than Nr (ANOVA: p=0.009). (B) Amount of chow consumed prior to blood glucose test (meal size) was nearly identical between supplemented and untreated mice in either genotype. Effects of age were not resolved.
Figure 2. Sugar-rich diet: rodent chow plus *ad libitum* sugar (Tg mice only; n=22; ages: 6–10 months). Average (non-fasting) daytime blood glucose levels were 22% lower in supplemented males compared to untreated males (ANOVA post-hoc SNK: *p=0.012*). Impacts of DSP not resolved in females. Untreated females had a 17% lower average daytime glucose compared to untreated males (*p=0.019*). Effects of treatment and sex were not resolved for measures of fasting (6 hrs) glucose and post-meal (30 min) glucose.
Figure 3. (A) Daily food consumption (standard rodent chow) per unit of body mass for supplemented and untreated Nr males and females (n=36), and supplemented and untreated Tg males and females (n=27). Differences in food consumption were not resolved between sexes and treatments in either genotype. Untreated Tg males consumed 52% less food per unit of body mass than untreated Nr males (ANOVA; post-hoc SNK: *p<0.01). Overall effect of genotype was significantly resolved (ANOVA: p<0.001). Ages: 6–18 months, no age-related effects. (B) Adult (ages: 8–16 months; n=18) Tg mice were fed both standard mouse chow and sugar cubes ad libitum. The ratios between sugar and chow consumption are shown per gram of body mass. Effects of DSP treatment were not resolved for female Tg (sugar preference was 49% and 41% for Unt and Supp females, respectively). Supplemented and untreated Tg males had a 63% and 43% preference for sugar, respectively (significant effect of treatment; ANOVA, post-hoc SNK: *p=0.015). Compared to untreated males, this represented a 46% increase. Sugar preference of supplemented males was also higher compared to all females.
Figure 4. Relationship between age and duration of spontaneous daily exercise for supplemented and untreated Nr (n=18) and supplemented and untreated Tg mice (n=22). All groups showed significant age-related declines in exercise duration with age, except for untreated Tg, who exercised very little across all ages. Linear regressions:

Nr Unt: $y=2.175-0.080 \times x; r^2=0.495; p=0.035$

Nr Supp: $y=3.043-0.095 \times x; r^2=0.640; p=0.001$

Tg Unt: $y=0.246-0.009 \times x; r^2=0.067; p=0.444$

Tg Supp: $y=1.552-0.109 \times x; r^2=0.492; p=0.016$

Mean exercise duration averaged over ages was 1.1 and 1.9 hrs, for untreated and supplemented mice, respectively (73% increase). ANCOVA (covariate=age) resolved a significant difference ($p<0.05$). Supplemented Tg showed a significant increase in exercise in youth but not in old age. ANCOVA (covariate=age) resolved a significant difference in slopes of age related regressions ($p<0.04$). Mean exercise duration for untreated and supplemented transgenic mice was 0.2 and 0.6 hrs/day, respectively.
Figure 5. Duration of immobility (excluding sleep) in time budget arena over 24 hours for (A) Nr supplemented and untreated (n=18) and (B) Tg supplemented and untreated mice (n=22) with respect to age. Untreated Nr had a 90% increase in duration of immobility between 4 and 24 months (age-related regression: \( p<0.05 \)). Supplemented Nr showed virtually no difference in duration of immobility across all ages. At 24 months duration of immobility for supplemented normal mice was 55% less than that of untreated counterparts. ANCOVA (covariate=age) resolved a significant effect of treatment (\( p<0.03 \)). Age related trends and effects of treatment were not resolved for Tg.
Figure 6. Sleep duration of Nr supplemented (n=9) and untreated (n=9), and Tg supplemented (n=11) and untreated (n=11) mice measured over 24 hours (ages: 4–26 months). No significant age-related effects were established. A 2.5 hour (19%) reduction in sleep was seen in supplemented Nr mice compared to untreated Nr (ANOVA; post-hoc SNK: **p<0.01). A 1.7 hour (12%) reduction in sleep duration was observed in Tg mice (ANOVA; post-hoc SNK: *p<0.05)
Figure 7. Duration of grooming and paper shredding for (A) supplemented and untreated Nr mice (n=17) and (B) supplemented and untreated Tg mice (n=21) with respect to age. For Nr mice, behavior did not show significant age-related regression. Mean duration of grooming/shredding was higher in supplemented Nr by 52% compared to untreated controls; effect of diet significant (ANOVA: p<0.006). For Tg, significant age-related decrease in duration of grooming/shredding was seen in the untreated group (r^2=0.43; p=0.027). Age-related increase for supplemented Tg was not resolved (p=0.210). Slopes of age-related regression lines were significantly different between treatments for Tg mice (homogeneity-of-slopes ANCOVA, covariate=age; p<0.05). Duration of grooming/shredding was identical for both treatments at ~6 months; but supplemented Tg showed an 80% higher duration by ~15 months of age compared to the untreated group.
5.8 REFERENCES


[40] World Health Organization. *Diabetes*. Fact Sheet Nº312; March 2013


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5.9 APPENDIX

**Figure A1.** View from above: Behavioral time budget arena with mouse (A) daytime and (B) night (full darkness; image recorded with infrared camera). Four compartments containing a running wheel, food dish, water nozzle and jar (nest) are connected via a central area. Top covered with wire mesh for climbing. Mouse was acclimated for 48 hours and videotaped (time lapse; 4 frames per second) for 24 hours. Durations of behaviors were manually scored at 1 second intervals from the recording.
Figure A2. Food consumption study: Pellets of rodent chow (12–14 pieces; Teklad® 8640) were glued vertically to the bottom of a small Petri dish (with non-toxic silicone). The smaller Petri dish was placed inside a larger one to collect crumbs. (A) Showing food dish before the experiment and (B) showing food 5 days after. Weight of food dish before and after was compared
CHAPTER 6

Chapter introduction:

I attempted to write this chapter as a short, high-impact paper. Somewhat different from previous works, these results are limited to middle-aged mice. We show that supplementation enhanced long-term memory retention in a challenging object recognition task. Age-related cognitive decline and the associated human conditions were previously introduced and discussed in Chapter 2. However, since each chapter is written as a stand-alone article, some repetition of general concepts here was necessary.

In this chapter we expand histological analyses to new regions of the brain. Chapter 3 documented findings in the cerebellum and the olfactory bulb. Here we show impacts of the DSP on neuronal numbers and markers of neuronal atrophy in the hippocampus and striatum. Current understanding of involvement of these regions in memory function is reviewed. Histological work was facilitated through collaboration with Dr. Sergei Aksenov, Dr. William H. Rodgers and Ted A. Aristilde from New York Hospital Queens (Flushing, NY). Also, collaborating with Chiara Nicolini (McMaster University) we quantified amounts of postsynaptic density protein 95 in cortical regions of the brain. The contribution of this scaffolding protein to synaptic plasticity and synapse stabilization and its role in learning and memory are reviewed.

Effects of aging on patterns of neuronal loss specific to the striatum and hippocampus along with age-related trends in postsynaptic density are presented. Potential human applications are addressed with emphasis on cognitive aging and neurodegenerative disease.

Note: Citation number [72] refers to Chapter 1; and citation number [1] refers to Chapter 2
CHAPTER 6

TITLE

A Complex Dietary Supplement Enhances Object Recognition Memory in Middle-Aged Mice in Association with Higher Neuronal and Synaptic Density

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6.1 INTRODUCTION

Cognitive decline is evident in aging animals [1–6] although onset of learning and memory deficits may begin even in early adulthood [5,7]. Accumulation of oxidative damage is commonly implicated [1,2,4,8]. Greater social interaction [9,10], enriched environments [10–12] and diets [1,13–16] may be offsetting. Different types of learning and memory tasks are differentially affected by aging [17]. Object recognition is the ability to discriminate between objects that were previously encountered compared to unfamiliar ones. Rodents exhibit a natural tendency to explore novel objects more, which allows assessment of recognition memory [18]. Compared to other cognitive tasks, recognition memory is less affected by age-related declines in sensory modalities and diminished motivation [17].

Earlier works suggested that recognition memory likely required hippocampal function and associated neural connections [19–22]. Recent studies suggested that integrity of the
hippocampus was not necessary for discriminating novel objects [23–27] (even after long (24h–3wk) retention intervals [28]); however, some studies in rats disagreed [29,30]. Latest findings argue that the hippocampus is indeed involved in learning and memory under normal conditions, as memory acquired prior to hippocampal damage is subsequently impaired [31–33]. However, if hippocampal lesions occur prior to learning, other brain regions may compensate to support acquisition of new memory [31,32,34].

Aging is a gradual and system-wide process that may not be accurately modeled by acute lesions to isolated brain regions. Moreover, studies on effects of brain lesions on memory are virtually exclusive to young animals, but aging may reduce compensatory mechanisms offsetting hippocampal decay.

Here we report that middle-aged C57BL/6 mice (10–20 months old) were unable to discriminate between novel and familiar objects following 24 hour retention delay. Remarkably, age-matched mice fed a complex dietary supplement (designed to ameliorate five biomarkers of aging [35–37]) showed a clear preference for exploring novel objects over previously encountered objects (indicating superior memory).

Age-related decline in hippocampal function is well established [2,38–41] and correlates with age-related memory loss [6] and Alzheimer’s disease (AD) [42–44]. Hippocampal dysfunction is mainly associated with changes in functional features and organization such as synaptic connectivity, synaptic strength and neuronal firing rates [6,38,39,41]. Interestingly, aging and age-related memory impairment is not accompanied by significant neuronal loss in the pyramidal layer of hippocampus [40,45,46]; although other hippocampal subdivisions may show neuronal depletion with aging [40]. In AD, however, distinctive neuronal loss is seen in CA1 pyramidal neurons [44]. Consistent with the above, we did not observe neuronal loss or damage in the pyramidal cell layer of middle-aged mice. Remarkably, however, higher counts of pyramidal neurons in the CA1 hippocampal region of supplemented mice were observed. While our mice do exhibit cognitive impairment at middle-to-old ages in a spatial learning task [1] the present findings appear to reflect memory enhancement rather than simple prevention of age-related memory loss (see: Discussion).
The striatum is highlighted in memory and learning [34,47–52] (although this is generally less emphasized than hippocampal aspects). Vulnerability of striatal neurons increases with age and neuronal loss in striatal regions is evident in normal aging [53–55] in association with cognitive decline [51]. Neuronal atrophy in striatum is linked to dementia [52], Parkinson’s (PD) [53] and Huntington’s disease (HD) [56,57]. Initial neuronal activity during learning appears in the hippocampus, but subsequently spreads to the striatum [48]. This may facilitate pattern formation for executable actions pertaining to the learned task [48] and reinforcement of memory connections [50]. Additionally, long term potentiation in striatum may be necessary for memory retention for periods of 24 hours or more [47]. One recent report claims that lesions to the striatum impaired object discrimination performance to a much greater extent than hippocampal lesions [34]. We found greater representation of normal neurons over atrophic neurons in striatal sections of supplemented mice. Reduced neuronal loss is consistent with better object recognition memory following a 24 hour retention interval.

The aging brain undergoes a decline in synaptic density [58–60] which may not necessarily involve or reflect atrophy of neuronal soma. Loss of synaptic density is implicated in cognitive deficits and AD [59–62]. Postsynaptic density (PSD)-95 belongs to a family of postsynaptic scaffolding proteins closely associated with synaptic plasticity, synapse stabilization and maturation, and long-term potentiation [63–66]. PSD-95 regulates synapse formation and synaptic strength (synaptogenesis) [67–69]. Lack of PSD-95 severely impairs learning [63] and decreased levels are directly linked to AD [70,71]. Levels of PSD-95 were higher in the frontal cortex of middle-aged supplemented mice (which may reflect synaptic status throughout the brain). This likely contributes to the observed enhancement in cognitive function.

6.2 METHODS

**Animals:** Random bred C57BL/6×SJL mice were housed in standard plastic cages with *ad libitum* food/water (room temperature: 23±2°C; light/dark photoperiod: 12/12h). All husbandry protocols adhered to CCAC specifications.
Complex dietary supplementation: A complex dietary supplement (DSP) was designed to target five mechanisms of aging [37,72]. Mice were randomly assigned to the supplemented or control group at weaning and maintained for life. Supplemented mice received one daily dose of DSP in home cages [1,72]. Composition of the DSP was described in [Ch. 4] and preparation and administration was explained in [72]. Biological targets of ingredients are found in [36; Ch. 4].

Object recognition: Mice were singly placed in a Plexiglas arena (120×80×60cm L×W×H) with wood-chip bedding on floor and water/food ad libitum. After 2 hours acclimation three plastic objects (different shape but roughly same size) (Fig. 1A) were placed and secured to floor at one end of the arena. Mouse was allowed to explore the objects for 24 hours. The objects were then removed, washed with soap and ethanol and reintroduced 24 hours later to their original positions, except that one of the objects was replaced by a novel object. Arena was videotaped from above and recording was later analyzed. Duration of exploring (sniffing, biting, mounting) (Fig. 1C) was scored over 10 minutes. The duration of exploring the two familiar objects was averaged and compared to novel object exploratory duration. Objects and positions were randomly rotated between different runs. A total of 10 supplemented and 9 untreated mice were tested (ages: 10–20 months).

Brain tissue preparation: Mice aged 12–28 months (different from those used above) were decapitated and brains removed. A section of the frontal cortex (~50mg) was sliced, frozen in liquid nitrogen and stored at -80°C. Cortices were used for analysis of PSD-95 (see below). The rest of the brain was stored in 10% neutral buffered formalin. Later, brains were processed, paraffin embedded, sliced at 5 microns and slides were prepared with H&E and Nissl stains as previously described [Ch. 3]. Neurons were counted in hippocampal pyramidal cell layer CA1, CA2 and CA3 (Bregma -1.50mm; Fig. 2A) and in sections of striatum (Bregma 1.10mm; Fig. 3A). In striatum: normal, early-stage atrophic and pyknotic neurons were identified from H&E stained slides based on visual criteria found in [52] and shown in Figures 3B and 3C. In the hippocampus: pyramidal neurons in CA1, CA2 and CA3 were discriminated as shown in Figure 2B (Nissl stain)
according to visual properties illustrated in (Figs. 4A and B in [73]; Nissl stain) and in (Fig. 4 in [74]; Nissl and H&E stain). Only middle aged mice were assessed (ages: 14–17 months; n=6 per group).

Concentrations of PSD-95 reflect synaptogenesis. Note that PSD-95 levels and neuronal assessment were done in different brain sections (see above). Results must be interpreted accordingly. Tissues were available for middle aged mice (12–20 months; untreated: n=10; supplemented n=8) and old mice (20–28 months; untreated n=8; supplemented n=10). Middle-aged mice were analyzed as a separate group to keep age ranges consistent with behavioral and histology data. PSD-95 levels for old mice are also presented.

**Protein extraction:** Approximately 50mg of tissue was homogenized on ice without thawing in homogenization buffer [see: 75] using a dismembrator. Homogenates were incubated on ice (15 min), centrifuged (12000×g, 20 min, 4ºC), supernatants were collected, and protein concentrations were determined using a DC protein assay kit (Bio-Rad Labs, Mississauga, ON, Canada).

**Western blotting:** Procedures described in [75,76] were adopted with minor modifications. Briefly, protein samples (35 µg) were resolved in 8% SDS-polyacrylamide gels, transferred onto PDVF membranes for 1.5hrs (250mA; 4ºC), blocked for 1h in 1:1 PBS/Blocking buffer (Cederlane no. 927-40000) at room temperature, and incubated with PSD-95 mouse antibodies (dilution 1:1000; Millipore) and β-actin antibodies (dilution 1:5000; Sigma) overnight at 4ºC. Then, membranes were washed [76] and incubated with secondary antibodies (IRDye 680 goat anti-rabbit and IRDye 800CW goat anti-mouse; diluted 1:8000; Licor Odyssey Biosciences, Lincoln, NE, USA) for 1h at room temperature. Detection and band measurement are described in [75,76]. PSD-95 values were normalized to β-actin for each sample.

**Statistical analyses:** Effect of DSP treatment on exploratory duration of familiar/novel objects was tested with ANOVA (post-hoc Duncan’s test), as effects of age were non-significant. Cortical PSD-95 was compared with ANOVA (post-hoc Duncan’s test)
between supplemented and untreated middle-aged and old mice. Neuronal count analyses employed a t-test.

6.3 RESULTS

**Object recognition:** No difference in exploration of familiar objects versus the novel object was found in untreated mice, indicating lack of memory retention (i.e. all objects were perceived as unfamiliar). Supplemented mice explored the novel object 75% longer compared to familiar objects (p=0.029; Fig. 1B). Relative lack of interest towards familiar objects suggests that memory of prior encounter was retained after 24 hours. Novel object exploration by supplemented mice was 70% higher compared to novel object exploration by untreated animals (p=0.044; Fig. 1A) indicating significant impacts of DSP treatment on memory retention.

**Hippocampus:** The number of pyramidal neurons in the hippocampus was augmented by DSP treatment. A 21% increase in CA1 pyramidal neurons in supplemented mice compared to controls was significantly resolved (p<0.05; Figs. 2B,C). CA2 and CA3 neuronal counts were 7% and 15% higher in supplemented mice, but this was not significant (Fig. 2C). Markers of neuronal damage or atrophy were not observed in either group. Indeed, normal aging is not associated with significant loss of pyramidal neurons [40,45,46]. Hence, these results suggest that in the hippocampus, supplementation likely promoted neurogenesis as opposed to preventing atrophy.

**Striatum:** Neuronal counts in striatum were assessed in a representative 240µm² area (see: Figs. 3A–C). Number of normal (i.e. healthy appearing) neurons was 14% higher in supplemented mice (p=0.019; Fig. 3D). The number of early-stage atrophic neurons was decreased by 25% (p=0.008; Fig. 3E). Pyknotic neurons were less frequent and similar in number between treatment groups (Fig. 3G). The ratio between degenerated neurons (early-stage atrophic + pyknotic) and normal appearing neurons was 19% lower in supplemented mice compared to untreated controls (p=0.013; Fig 3F). Higher count of healthy neurons and lesser count of atrophic neurons are suggestive of alleviated neurodegeneration in striatum.
**Postsynaptic densities:** PSD-95 protein concentrations in cortical sections of middle-aged mice (12–20 months) were over twofold higher in the supplemented group (p=0.043; Fig 4A). High variability (error bars) suggests individual variation in responses to supplementation. A portion of mice showed little change in PSD-95 from controls, while others had markedly elevated values. Effects of supplementation on PSD-95 levels were not obtained in old mice (>20 months) (Fig. 4A). No other group combinations were resolved. Higher post synaptic density is consistent with better memory function, although the present findings only reflect the cortex and not the striatum or hippocampus.

6.4 DISCUSSION

We previously reported that spatial learning in the Morris water maze (MWM) was impaired in mice aged 12–25 months, but youthful learning ability was entirely preserved in age-matched DSP supplemented animals [1]. Here, we document effects of DSP treatment on cognitive performance in a non-spatial memory task for younger cohorts of mice (10–20 months old; middle-ages). This is a challenging test for mice and middle-aged untreated animals were unable to discriminate between novel/familiar objects following 24h retention. Conversely, supplemented mice showed a clear preference for exploring the novel object (Fig. 1B) indicative of enhanced retention of recognition memory. In contrast to other studies [23,77], we used objects that were more difficult to discriminate (i.e. similar shape and size; see: Fig. 1A) and employed a longer retention interval (24h) compared to that of a few minutes commonly tested. Also, mice were allowed to interact with objects for an entire day prior to withdrawal, ensuring thorough familiarity. While more laborious, this protocol obtained clear discrimination of memory function specific to long-term memory retention.

Spatial learning (e.g. MWM) combines hippocampal and cerebellar processing [78; Ch. 3]. The cerebellum ages sooner than the hippocampus [79], suggesting that spatial learning deficits may prevail despite normal hippocampal function [80]. Object recognition is not linked to the cerebellum, however some evidence suggest a possible role of this region in non-motor memory [81,82]. DSP augmented cerebellar neuronal
numbers and associated behaviors [Ch. 3] suggesting that improvement in the MWM may at least partly reflect improved cerebellar function.

Enhanced performance in the object recognition task suggests that impacts of the DSP directly extended to hippocampal function, as normally, recognition memory is linked to this region [29–33] (see: Introduction). We also show that counts of pyramidal neurons were significantly higher in the CA1 hippocampal region (Fig. 2C) consistent with better memory retention.

The CA1 region of the hippocampus is particularly vulnerable to stress, exhibiting most neuronal loss in ischemia [83,84] and AD [44]. Damage to the CA1 region impairs object recognition memory in rats [83]. In normal aging, however, loss of pyramidal neurons is minor [40,45,46] so little decrease is expected in middle-aged mice. Cell damage was not visible in pyramidal neurons of untreated mice suggesting that higher CA1 neuronal numbers in supplemented animals (Fig. 2) likely reflected neurogenesis, as opposed to age-related loss in untreated mice. Adult neurogenesis is documented in the hippocampus for rodents and primates [84–96] and a positive link between new cell numbers and learning is reported [90,96]. Continuous neurogenesis is well documented in granule cells in the dentate gyrus hippocampal region [84,95] (presently not assessed in our mice). In comparison, CA1 pyramidal neurons appear to have limited regeneration [94,95].

However, at least two studies confirmed significantly detectable formation of new pyramidal neurons (neurogenesis) specifically in the CA1 region following ischemic hippocampal injury [84,96]. Despite limited evidence, these examples indicate that the adult hippocampus retains the capacity to generate new pyramidal neurons under certain conditions. Our findings (21% increase in number of CA1 neurons in supplemented mice; p<0.05; Fig. 2C) are most consistent with DSP-induced neurogenesis; however, conclusive validation is pending analysis with Bromodeoxyuridine (BrdU) stain.

Modest increases in hippocampal CA2 and CA3 neurons (7% and 15%, respectively) in supplemented mice indicate that impacts of DSP may not be limited to the CA1 region;

BrdU staining is employed specifically for in vivo detection of proliferating cells including neurons (neurogenesis)
however neuronal counts in these regions were not significantly resolved between treatments (Fig. 2).

Positive regulators of neurogenesis such as enriched environments [97], estrogen [98] and physical exercise [85] greatly enhance hippocampal learning. Voluntary exercise (wheel running) in mice was directly linked to increased hippocampal neurogenesis in adult [85,86,88,89] and old mice [87], and enhanced performance on the MWM [85,87]. However, in some cases improved learning did not reflect hippocampal neurogenesis [89]. Our DSP was shown to elevate spontaneous locomotion [72] and spontaneous exercise [Ch. 5] in mice across ages. Spatial (MWM) [1] and non-spatial (objects recognition) (Fig. 1B) forms of leaning were also augmented. Detection of higher neuronal numbers here is suggestive of augmented adult neurogenesis. Oxidative damage, inflammatory processes and general stress (marked by upregulated stress axis and circulating glucocorticoids) impair hippocampal learning, memory [92,99–101] and neurogenesis [93,102]. Antioxidant approaches may be offsetting [102]. We previously documented significantly reduced protein carbonylation [72] and nitration [103], as well as ameliorated symptoms of systemic inflammation (unpublished) in supplemented mice. In addition, we suggest mitigation of the stress axis by the DSP [Ch. 3]. All these aspects are likely to promote neurogenesis. BrdU staining would obtain further confirmation.

Given that marked cognitive impairment is not expected in middle-aged mice, it is possible that lack of memory retention in the object recognition task was not a consequence of age-related decline. The challenging nature of our protocol (e.g. similar objects, long retention interval) may simply exceed the cognitive capacity of mice. In that case, our findings constitute the first evidence for memory enhancement by DSP (as opposed to our previously reported cognitive preservation in old age [1]). There are few documented treatments that can elevate endogenous cognitive/memory functions other than those promoting increased activity (notably sugar, caffeine and nicotine). Consequently potential implications of DSP impacts on both brain function and microstructure extent far beyond cognitive aging.
Striatal neurons majorly contribute to recognition/discrimination memory [34,47–52]. In some cases the striatum may play a greater role in recognition memory as compared to the hippocampus [34]. Long-term memory (≥24h) [47] and reinforcement of memory connections (via continued stimulation) [50] are attributed to the striatum. This further emphasizes striatal involvement in the current memory task as mice were (a) allowed 24 hours to interact with objects prior to withdrawal, and (b) a 24 hours retention interval was employed.

We found that the population of normal neurons was 14% greater in the striatum of middle-aged supplemented mice, while the number of early-stage atrophic neurons was reduced by 25% (Fig. 3). Striatal neurons are sensitive to aging and related stress [53–55], and our findings suggest neuroprotective effects of the DSP. Neuronal loss in the striatum is associated with poor memory such as in our control mice, whereas enhanced memory retention in supplemented animals is consistent with higher neuronal numbers. Total number of neurons (damaged + healthy) was similar in supplemented mice and controls. While adult striatal neurogenesis is possible [104,105], effects of the DSP on striatum appear neuroprotective rather than neurogenic.

Mitochondrial dysfunction is particularly highlighted in striatal neuronal loss [54,55]. We showed higher whole-brain mitochondrial activity in supplemented mice associated with enhanced locomotion [72] and spatial learning [1]. Greatest impacts on mitochondrial function were observed at older ages (>1.5y) [1,72; Ch. 4], while middle-aged mice experienced smaller elevation. Nonetheless, the associated improvements in functional aging are apparent in adult ages (Fig. 1B) [72, Ch. 3–5].

Synaptic deficits are associated with cognitive impairment [59–62,70,71]. Synaptic density declines with age [58–60] as reflected by concentrations of PSD-95, a scaffold protein marker of synaptic density and synaptogenesis [65–67]. PSD-95 is directly implicated in long-term potentiation and learning [63] and exhibits sensitivity to oxidative stress [106]. PSD-95 levels in middle-aged supplemented mice were twofold higher than in controls (Fig. 4); however, this was quantified only in sections of frontal cortex. Impacts of the DSP on synaptic densities in the hippocampus and striatum remain
to be assessed. Object recognition was not impaired in rats with frontal cortex lesions [23] suggesting that this regions does not participate in recognition memory. Therefore, enhanced memory of supplemented mice may not be linked with higher PSD-95 levels at present. However, the DSP augmented structural morphology and/or neuronal populations in all brain areas examined (e.g. hippocampus: Fig. 2; striatum: Fig. 3; cerebellum and olfactory bulb: [Ch. 3]), suggesting that benefits of treatment are likely general to whole brain.

Our findings suggest that complex dietary interventions may have sizable benefits for memory enhancement and neuronal status even in middle-aged brains. This has strong implication for aging and age-related pathologies such as AD [42–44,62,70,71], PD [53,107] and HD [56,57]. Augmented hippocampal neurogenesis promises cognitive benefits independent of anti-aging contributions. Human trials are being initiated to confirm translation of benefits to humans.

6.5 ACKNOWLEDGMENTS

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6.6 FIGURES and TABLES

Figure 1. (A) Objects used in the object recognition study. Objects {a/c}; and {b/d} were not exchangeable as familiar/novel pair as they are too similar. A typical run employed {a,b,c} replaced with {a,e,c}. Objects were always returned in the same orientation following 24h retention. (B) Duration of exploring familiar versus novel object for supplemented (n=10) and untreated (n=9) mice (ages: 10–20 months). No significant difference was observed in exploration of objects in control mice. Supplemented mice showed a 75% longer exploratory duration of the novel object compared to the familiar one (ANOVA: post-hoc Duncan’s test: *p=0.029). Novel object exploration was higher in supplemented mice compared to control mice (*p=0.044). (C) Mouse exploring object, typical behavior involved direct contact.
Figure 2. See caption on next page
Figure 2. (From previous page) (A) Cross section of hippocampus showing CA1, CA2 and CA3 pyramidal cell layer areas (×40 magnification; Nissl stain; Bregma -1.50mm). (B) Close up showing organization of pyramidal layer area CA1 (×200 magnification, control mouse). Cells were easily differentiated; elongated dark cells and pyramidal cells without visible nucleoli (arrow) were not counted. (C) Supplemented mice showed 21% higher cell number in CA1 compared to untreated controls (t-test: *p<0.05). A 7% and 15% higher cell number in supplemented mice (areas CA2 and CA3, respectively), was not statistically resolved (p=0.591; p=0.219). Mice aged: 14–17 months; n=6 per group; no sex differences
Figure 3. See caption on next page
Figure 3. (From previous page) (A) H&E stain brain cross section (×40 magnification; Bregma 1.10mm) showing striatum (caudate putamen; CPu) and location of representative section (240µm²) used for neuronal count. Detail of untreated (B) and supplemented (C) striatal area showing normal neuronal cells, N; early-stage atrophic neurons, solid arrows; and end-stage pyknotic neurons, open arrows (×400 magnification). Notice the relative scarcity of normal cells and higher number of degenerated neurons in untreated striatum. (D) Total number of normal neuronal cells, (E) early-stage atrophic neurons, and (G) pyknotic neurons. Compared to untreated controls, supplemented mice showed a 14% (*p=0.019); increase in normal neuronal population, a 25% (**p=0.008) decrease in early stage-atrophic neurons and a nearly identical numbers of pyknotic neurons. (F) Shows the ratio of degenerated neurons (early-stage + pyknotic) to normal neurons. A 19% difference in ratio was significantly resolved (*p=0.013). Ages: 14–17 months; no effects of sex; all groups compared with a t-test; n=6 per group
Figure 4. Quantification of PSD-95 protein in frontal cortex homogenates of middle-aged (10–20 months; n=18) and old (>20 months; n=18) mice. PSD-95 levels were 2.2 times higher in supplemented middle-aged mice compared to untreated controls (ANOVA post-hoc Duncan’s test: *p<0.05). No differences were seen in old mice or between age groups; genders were not resolved.
6.7 REFERENCES


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CHAPTER 7

Chapter introduction:

Applications of our DSP for prevention and treatment of human neurodegenerative disease, diabetes and age-related macular degeneration were suggested in previous chapters. This chapter examines impacts on the DSP on cancer rates. Our DSP was not specifically designed as an anti-cancer formulation. However, because aging is a major risk factor, slowing aging and offsetting age-related cellular processes should ultimately impact cancer. We added five new ingredients to the original DSP formula in order to cover a greater list of targets specific to tumorigenesis.

The majority of normal mice never develop cancer in their lifetimes. Therefore, we used p53+/- mice which exhibit high incidence of spontaneous tumorigenesis. Understanding of tumor growth gained from these mice in relation to human cancers is addressed. The action of the p53 tumor suppressor gene is described under normal conditions and stress, and its role in aging is highlighted.

In this chapter we document a significant 30% decline in cancer incidence attributed to supplementation. Greatest impacts were seen in lung cancer which is the leading cause of cancer related mortality in humans. Metastases were undetectable in supplemented mice but occurred in untreated animals.

Effectiveness of anti-aging interventions necessitates incorporated relief from pathologies that may result in premature death. In humans, the risk of developing cancer rises steeply after the sixth decade. Therefore, establishing impacts of the DSP on rates of spontaneous tumorigenesis was imperative to our research direction. Impacts of supplementation on longevity and body mass are also presented.

Note: Citation number [19] refers to Chapter 1; and citation number [20] refers to Chapter 2
CHAPTER 7

TITLE

Effects of Complex Dietary Supplementation on Tumorigenesis in p53+- Mice

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7.1 INTRODUCTION

The majority of human cancers are linked to mutation or loss of the p53 tumor suppressor gene [1–6]. Deficiencies in p53 cell signal pathways are estimated in 50–80\% of tumors affecting humans [2,7], and \textit{p53} mutations can affect over fifty cell types and tissues [4]. The p53 pathway is recognized as a crucial factor in preventing tumorigenesis in humans and mice [2,8,9].

The p53 protein functions to permissively allow cell cycle progression, induce cell cycle arrest to allow for repair, or initiate apoptosis if damage is severe [6,9]. It responds to diverse stressors including nutrient deprivation, hypoxia, oncogene activation and DNA damage [6,9]. Failure in p53 function can lead to unchecked proliferation of damaged cells resulting in cancer [2,9]. Tumor suppression depends on the amount of p53 expression; therefore, the number of p53 gene copies is instrumental (i.e. haploid vs. diploid) [5,8–11]. Additionally, emerging implications of p53 in cell senescence reveal important tradeoffs between tumor suppression and mammalian aging [2,6,9,11].

Mouse models with deficient p53 function are useful in the study of tumor development. Nulizygous (p53-/-) mice succumb to spontaneous tumors by 10 months of age resulting in drastically reduced lifespan [8]. In contrast, haplodeficiency yields a
milder phenotype. p53+/- heterozygous mice exhibit delayed onset of tumorigenesis manifesting at 1–2 years of age, but some mice may not develop tumors at all [8]. Maximal longevity is comparable with that of p53+/- controls (~3 years); however, mean survivorship is reduced with upwards of 90% of p53+/- mice dying by 2 years of age [8]. Compared to nullizygotes, p53+/- mice are a better model for testing potential interventions. Complete loss of p53 results in rapid and aggressive tumorigenesis unlikely to respond to most potential interventions. On the other hand, p53+/- mice display less acute syndromes increasing likelihood of effective intervention. Delayed onset also allows for a longer course of treatment.

Cancer is the second leading cause of death in developed nations [12–14]. Over 1.6 million new cases and over half a million cancer deaths were projected in the US for 2012 alone [14]. The probability of developing cancer strongly increases with age with ~80% of cancer-related mortality in patients over 60 years old [14]. We found that cancer incidence was also much greatest in older cohorts of p53+/- mice indicating comparable age-related trends. There is also substantial overlap in the types of cancers commonly observed in humans and those found in p53+/- mice. The four cancers commonly affecting p53+/- mice are lymphomas, soft tissue sarcomas, osteosarcomas and carcinomas of lung [8,11]. Lymphomas and lung carcinomas were among the ten most frequently diagnosed human cancers in 2012 [14] and soft tissue sarcomas are also common [14]. One exception is osteosarcoma which frequently occurs in p53+/- mice, but rarely in aging humans.

Given the frequent dysregulation of p53 in human and murine cancers, the similarity in cancer types and comparable age-related trends, it appears that p53+/- mice are reasonably good models for understanding human cancer. Importantly, this also suggests that interventions benefiting p53+/- mice may translate to humans.

Application of dietary supplements for preventing cancer is a rapidly expanding field. Searching Google Scholar for: “dietary supplements” + cancer + prevention returns 609 articles published in 2000; 2,970 articles in 2010; and over 3,500 articles in 2012 alone. Agreement on the possible effectiveness of vitamins, micronutrients, minerals, herbal
extracts and other nutraceuticals for cancer prevention remains relatively inconclusive. Lack of consistency is exacerbated by highly variable experimental protocols, diverse confounding factors, narrow interests, combinations of treatments, and different criteria for determining effectiveness of intervention. Furthermore, many in vitro findings fail to translate to whole organisms.

Caloric restriction (CR) can be effective against tumorigenesis. Longo and Fontana [15] review studies on cancer prevention by CR dating back several decades. However, CR had little benefit on tumor incidence in p53+/- haplodeficient mice [16]. Regardless, CR requires relatively drastic fasting regimes that are challenging for most people to adopt.

In the present study we show that treatment with a complex dietary supplement (DSP) designed to target multiple biological pathways significantly reduced incidence of primary and metastatic tumors in p53+/- mice. Effectiveness of our DSP, where less complex formulations have generally failed, may reflect that it was targeted to multiple important mechanisms associated with tumorigenesis, and the opportunity for synergistic, complementary and emergent interaction effects.

Our DSP was originally formulated to target five key mechanisms of aging. Indeed, we showed that it extended lifespan and prevented age-related cognitive, sensory and motor decline in mice [17–21; Ch. 3–6]. Given the strong age-related etiology of cancer [6,9,14] the multiple-supplement approach was hypothesized to likely provide benefits. The original formulation [19] was modified to include an expanded list of targets specific to tumor development. Table 1 lists components of the anti-cancer DSP used here. Aside from adding five new ingredients we retained the previous composition virtually unchanged.

Most spontaneously arising tumors ultimately trace to genotoxic stress and resulting DNA damage [2,6,9]. The likelihood of cells incurring DNA damage is independent of p53 levels. Rather, p53 serves to arrest proliferation of damaged cells, and provides time for possible repair (including DNA) [2,6,9]. Given the function of p53, minimizing DNA damage should forestall tumorigenesis regardless of p53 status. We previously reported that our DSP completely protected DNA from damage by ionizing radiation [22]. This
suggests that the DSP could offset tumorigenesis in p53+/- mice despite their haplodeficiency. In addition, effects of DSP on activation of p53 are also possible.

This work examines effects of our DSP on rates of spontaneous tumorigenesis in p53+/- mice with respect to commonly occurring tumor types. We identify biological targets of our DSP in relation to mechanisms involved in primary tumor formation and metastatic growth. Possible mechanisms of cancer prevention are proposed and evaluated in light of recent literature. Impacts of the DSP on survivorship and body mass are also described with attention to growth factors and their role in cancer development. In addition, regulatory impacts of p53 on aging, and emerging tradeoffs between aging and tumor suppression are considered. Finally, a brief overview of literature is provided outlining the biological actions of components of our DSP in relation to prevention and progression of cancer.

7.2 METHODS

Animals: A total of 114 male p53+/- mice aged ~3 months were donated by Dr. Douglas R Boreham, McMaster University. Mice were maintained to endpoint or natural death. All husbandry protocols adhered to the Canadian Council for Animal Care standards. Mice were housed up to three siblings per cage. Aggression between cage-mates was rare and mice were separated at the first instance of aggressive behavior, if displayed. Standard rodent chow (Teklad® 8640) and water was provided ad libitum. Animal rooms were at 23±2ºC and 12/12 hours light/dark cycle.

Complex dietary supplement and treatment protocol: Table 1 lists components and dosages of the complex dietary supplement (DSP). For the present study we modified the original formulation [19] adding five new ingredients to better impact targets associated with tumorigenesis. Table 3 highlights the biological functions of the newly added components relevant to cancer prevention. The DSP is composed of vitamins, minerals, herbal extracts, micronutrients and other nutraceuticals available for human consumptions without prescription at most health food stores. Mouse dosages were calculated based on recommended human doses adjusted for smaller body size and higher
metabolic rates of mice as described in [17]. The DSP was prepared by mixing all the ingredients in a small amount of distilled water and pipetting the resulting slurry onto small square pieces of bagel. Supplemented mice received one bagel square once a day in home cages. Untreated mice received a plain bagel square of the same size.

Of the 114 mice 60 were randomly assigned to the supplemented group while 54 remained untreated. In previous studies we began treatment from weaning [17–21]. Presently, mice were obtained at ages between 75 and 115 days (mean age: 98 days) and supplementation began immediately thereafter. Our experience shows that when introduced at adult ages mice are initially reluctant to eat the entire bagel square with a full dose of diet. Therefore, we began by feeding mice a plain bagel square (which they consumed avidly) followed by gradually increasing the amount of DSP soaked onto the bagel square over a 3 months period to full dose. As a result, supplementation with a full dose was not achieved until ages of 165 to 205 days (i.e. ~6 months of age).

**Dietary consumption index:** Despite gradual acclimation some mice did not fully ingest the bagel square on a consistent basis. To account for quantity of DSP consumed by each mouse we checked for uneaten remnants following daily feeding sessions. If found, the weight of the uneaten piece was subtracted from that of the full square. We compiled a consumption index reflecting lifetime DSP consumption for each mouse given as a score between 1 and 0 (where 1 indicates 100% lifetime consumption; 0.75 indicates 75% consumption; etc). Longevity was compared to the DSP consumption index to determine whether lifespan was dependent on amount of DSP consumed.

**Longevity:** Longevity was assessed by compiling survivorship curves for the entire populations of untreated and supplemented mice. Animals found dead or those culled due to tumor endpoints were included in the survivorship analysis. Several animals succumbed to skin lesions at young ages unrelated to tumor development. These animals were culled and excluded from survivorship analysis.

**Body mass:** Mice were periodically weighed between 75 to 500 days of age. Each mouse was weighed on ten occasions unless died prior to 500 days. Body mass of
supplemented and untreated animals was compared across ages (from repeated measures). Lifetime average mass was also compared between groups.

Pathology: Mice were sacrificed at endpoints that included: development of large tumor mass, limb paralysis, poor body condition, immobility, frailty, irregular breathing and prolapses. All masses, enlarged lymph nodes and tissues with obvious tumors were collected. Regardless of visible pathology we collected liver, spleen, lung, thymus, spinal column, brain, kidneys and adrenals for each animal to test for possible metastases. All tissues were placed in formalin and submitted to a pathologist for histopathological examination. Full pathological reports were completed on 41 of 54 untreated mice and 45 of 60 supplemented mice. Thirteen untreated mice (24% of population) and 15 supplemented mice (25% of sample) were not tested because we failed to collect tissues from those animals. Since proportions of untested animals in each group were virtually identical and resulted randomly, the untested animals did not constitute an experimental bias. Tumor types and frequencies of occurrence were compared between supplemented and untreated mice.

Statistical analyses: Frequencies of tumorigenesis in supplemented and untreated mice for each type of tumor were compared using a two-tailed chi-square (Fisher’s) test. Differences in survivorship between treatment groups were analyzed with a log-rank test. Body mass with respect to age was compared using ANCOVA (covariate=age). Mean body mass was compared using a t-test. Regression analysis was used to test the correlation between longevity and DSP consumption index. All analyses were performed using Statistica® 6.0 software.

7.3 RESULTS

Spontaneous tumorigenesis: The number of mice succumbing to tumors was significantly reduced by the DSP. Of 41 control mice 34 (83%) developed tumors compared to only 26 of 45 (58%) supplemented mice (30% reduction; p=0.02; Table 2). The predominant tumors observed in our mice were sarcomas, lymphomas and
carcinomas. Incidences of these tumors with respect to DSP treatment are shown in Table 2.

Sarcoma: Occurrence of sarcomas was most frequent, affecting 48% of animals. Osteosarcoma was found in 13 (32%) untreated and 8 (18%) supplemented mice. The 44% reduction in incidence of osteosarcomas in supplemented mice was not significantly resolved (p=0.208; Table 2). Osteosarcomas were predominantly observed in lumbar vertebrae, but occasionally the skull, jaw, liver, lung and sternum were affected. Soft tissue sarcomas were observed in 21% of mice. Of these, hemangiosarcoma affected three untreated and five supplemented mice. Two cases of fibrosarcoma were diagnosed in supplemented mice and one case in an untreated mouse. One case of histiocytic sarcoma affecting seven tissues was found in an untreated mouse. One case of astrosarcoma (brain) and one case of chondrosarcoma occurred in supplemented mice. No significant differences were resolved.

Carcinoma: Carcinoma affected 26% of animals and was diagnosed in 16 untreated and 6 supplemented mice (Table 2). DSP treatment resulted in a 67% decrease in carcinoma (p=0.031; Table 2). Other than two cases of basosquamous cell carcinoma in untreated mice, the rest were pulmonary adenocarcinomas. Frequency of pulmonary adenocarcinomas was 34% in untreated mice (14 of 41) compared to only 13% in supplemented mice (6 of 45). The 62% reduction was statistically resolved (p=0.039; Table 2). This indicates that supplemented mice experienced significantly reduced lung cancer.

Lymphoma: Lymphomas occurred in 16% of animals, affecting 5 supplemented and 9 untreated mice (Table 2). Due to limited sample size this ~50% reduction was not statistically resolved. In all but one case, lymphomas were extranodal affecting a wide range of tissues including lung, liver, spleen, kidneys and vertebrae. Often, multiple tissues and organs were affected in the same animal.

Pheochromocytoma: Two cases of pheochromocytoma were diagnosed in untreated mice. While this tumor was not diagnosed in supplemented animals, low incidence precluded statistical resolution.
Multiple tumor burden: Multiple tumor types of primary origin were found in 13 (32%) untreated mice; most frequently a combination of pulmonary adenocarcinoma and osteosarcoma. In two cases, three primary tumor types were present. Conversely, only four supplemented mice (9%) had multiple tumors (Table 2). A chi-square test confirmed that the incidence of multiple tumor burden was significantly lower in supplemented animals (p=0.013; Table 2).

Metastasis: Metastases were confirmed in 7 (17%) untreated mice. Remarkably, tumors of metastatic origin were absent in all 45 supplemented animals. This difference was strongly resolved (p=0.004; Table 2) indicating that the DSP limited spread of cancer cells to secondary sites.

Relationship between tumorigenesis and age: Mice sacrificed due to tumor-related endpoints were categorized into 50 day intervals (Fig. 1). The youngest mouse to develop a tumor was 249 days old. Between 350 and 600 days of age, roughly 2 to 6 mice were diagnosed with a tumor every 50 days. Frequency of tumorigenesis was virtually identical for supplemented and untreated groups prior to 600 days of age (see: Fig. 1). However, incidence of tumor endpoints quickly rose in >600 day old untreated mice, and by age >650 days tumor rates were significantly differentiated (chi^2: p<0.018; Fig. 1). Thus, benefits of the DSP strongly manifested in older ages.

Longevity: Analysis of survivorship curves revealed no impact of the DSP on longevity of p53+/- mice (Fig. 2) despite the marked reduction in tumor frequency (Table 2). Four of the longest lived animals were in fact untreated (Fig. 2), but this was not significantly resolved. Notably, these mice were tumor free.

Longevity and dietary supplementation: Survivorship data (Fig. 2) indicated that supplemented mice did not express an increase in maximal lifespan. However, longevity was linked to rates of DSP consumption. A DSP consumption index was assigned to every supplemented mouse (see: Methods). Two mice had a DSP consumption index of less than 0.6; 85% of mice had an index of 0.8 or better; and 62% and 41% had indices of >0.9 or >0.95, respectively. Plotting the longevity for each supplemented mouse against its consumption index revealed a strong positive relationship between longevity and
consumption of the DSP (p=0.0001; Fig. 3). The longest lived mice were those consistently ingesting the highest doses, whereas the shortest lived mice ate the least DSP (Fig. 3).

**Body mass:** We regularly recorded body mass from 75 to 500 days of age. Figure 4A illustrates that the growth rate of <300 day old mice was significantly higher in untreated compared to supplemented mice (ANCOVA, homogeneity-of-slopes analysis: p<0.002; Fig. 4A). This difference was small, however, amounting to a body mass difference of only 3.6% between treatment groups at 290 days of age. Comparison of mean adult body mass between treatments (mice >400 days old) was not statistically significant (t-test: p>0.25; Fig. 4B) indicating that despite a slower growth rate, supplement mice attained nearly identical adult body sizes.

7.4 DISCUSSION

**Tumorigenesis, dietary supplementation and cancer types:** DSP treatment reduced the number of p53+-/- mice succumbing to tumors by 30% (p=0.018, n=86; Table 2). The biggest decline (67%) was in carcinoma cases (p=0.031). Despite trends for other cancers, low sample sizes obscured statistical resolution. For example, lymphomas affected only 16% of mice and despite a 50% decline, impacts of the DSP were not resolved (Table 2). Pheochromocytomas occurred exclusively in untreated mice; however, effects of treatment were inconclusive as only two cases were diagnosed (Table 2). Sarcomas were more common, affecting 48% of animals, but were little affected by supplementation. Osteosarcomas, found in 24% of mice, were 44% less frequent in supplemented animals, but fell short of significance (p=0.208; Table 2). Overall, tumor protection by the DSP appears very promising given the positive trends. A larger sample size would yield better resolution.

**Cancer types in p53+-/- and normal mice:** Sarcomas, lymphomas and carcinomas collectively comprise over 95% of tumors spontaneously occurring in mice regardless of p53 status [8,11]. However, p53+-/- mice exhibit significantly different relative proportion of these tumors compared to p53+/+ controls. Specifically, sarcomas comprise 20% of
tumors in p53+/+ rodents, but are predominant in 53+-/ mice with a frequency of over 60% [8,11]. Our data support these trends (Table 2): loss of p53 function specifically promotes onset of sarcomas over other tumors. In fact, the frequency of lymphomas and carcinomas increases by only 2 to 4 times in p53+-/ mice compared to p53+/+ controls; whereas sarcoma frequency increases by 15 to 30 times [11].

Mechanisms involved in formation of specifically sarcomas often involve p53 inhibition. The product of the *MDM2* gene functionally downregulates p53 transcription mimicking inactivation of the *p53* gene [23]. In sarcomas *MDM2* may be amplified by up to 50 times [24]. Alternatively, lymphomas and carcinomas rarely exhibit *MDM2* amplification or overexpression [25–27].

Our DSP had the greatest impact on carcinomas and lymphomas, while impact on sarcomas was comparatively minor. Considering that normal animals exhibit a smaller proportion of sarcomas and larger proportions of carcinomas/lymphomas, means that impacts of the DSP on overall tumor incidence (i.e. combined tumor types) may be greater in normal (p53+/+) mice. Here we reported a 30% reduction in tumor rates, but this difference was weakened by a relatively high proportion of sarcomas which were poorly affected by the DSP.

**Lung cancer:** Lung cancer comprised only 14% of human cancer cases diagnosed in the US in 2012 yet it represents the second leading cause of death after heart disease [14]. Indeed, 26–29% of all cancer deaths are due to lung cancer which, once engaged, does not respond well to available treatments [14]. Pulmonary adenocarcinoma is the most frequent lung cancer worldwide and the relative proportion of such cancer is increasing [28]. Remarkably, DSP treatment resulted in a 62% reduction in pulmonary adenocarcinoma cases in our mice (p=0.039; Table 2). Regardless of results for other cancers this may be our most important finding given the high morbidity associated with this disease. Even modest translation of our results to humans would represent very strong clinical, economic and social benefits for preventative treatment of lung cancer.

**Tumorigenesis and oxidative stress:** Reactive oxygen and nitrogen species are closely linked to initiation and progression of cancer [29–32]. Many types of cancer cells display
redox imbalance reflecting elevated reactive oxygen species (ROS) [30]. In fact, stimulation of cell proliferation and growth require free radical generation [33]. Consequently, augmentation and/or enhanced activity of cellular antioxidant systems emerges as a likely candidate mechanism for prevention and amelioration [31,34–37]. Indeed, it was shown that reduced activity of enzymatic antioxidants (such as MnSOD) increased incidence of cancer [38]. Of the 35 ingredients in our DSP, 27 have antioxidant properties scavenging a full range of mitochondrial and cytoplasmic free radical species including \( \text{H}_2\text{O}_2 \), \( \text{O}_2^- \), \( \text{OH}^- \), \( \text{NO} \), \( \text{NO}^- \), \( \text{O}^- \) and \( \text{ONOO}^- \) [22]. Reduced tumorigenesis in supplemented p53+/− mice likely traces at least partially to superior antioxidant protection.

DNA mutations (primarily from oxidative damage) are pivotal to tumor initiation [30]. Markers of DNA damage such as chromosomal aberrations (CAs) and 8-OHdG (biomarker of oxidative base damage) are closely associated with cancer [30,31,39]. We previously reported that CAs and 8-OHdG levels in mice subjected to 2-Gy whole body radiation were significantly lower in supplemented mice compared to untreated controls [22]. Thus, the DSP offered substantial DNA protection from radiation-induced oxidative stress consistent with the present tumorigenesis data (Table 2). Furthermore, we showed that oxidative and nytrosative protein damage in brains of supplemented mice was also significantly reduced [19,21]. This is highly relevant to the present study as both oxidized and nitrated proteins are elevated in some cancers [32].

**Mitochondrial ROS:** Mitochondria are the primary generator of ROS [33,40]. Normally, superoxide radicals expelled during cellular respiration are removed by resident superoxide dismutases [40]. However, abnormal mitochondrial function may lead to unregulated release of free radicals. Age-related accumulation of damage can reduce concentrations of functional electron transport chain (ETC) complexes [19,20,41,42]. Deficiencies in ETC complexes not only lower energy output, but also increase ROS generation by disrupting flow of electron transfer [40]. This translates into chronic energy decline and higher oxidative stress sufficient to increase mutation rates. Therefore, maintaining (or upregulating) healthy mitochondrial function in aging should
reduce oxidative stress and associated damage to DNA, proteins, membranes and cellular organelles.

Several ingredients in our DSP support mitochondrial function [22]. We showed that the DSP increased activity of mitochondrial ETC complexes II–IV in aging mice in association with reduced oxidative damage [19–21; Ch. 4]. Increased lifespan [18] and improved cognitive and motor function were also observed [17,19,20]. Now we extend benefits of supplementation to reduced cancer rates. Improved mitochondrial function is highly implicated as a contributing factor.

Cells of long-lived calorically restricted (CR) mice have low levels of ROS [43,44]. This does not involve diminished oxygen consumption, but rather decreased electron flow directed to ROS generation [43,45]. In fact, CR organisms often show elevated mitochondrial activity [44]. Thus, the DSP mimics some aspects of the CR paradigm [19–21]. CR can also reduce tumor incidence [46] which likely involves some contribution from reduced mitochondrial ROS generation. Although diminished growth is a prominent association in CR animals, and likely contributes to reduced tumorigenesis, the small 3.6% reduction in early-life growth of supplemented mice is unlikely to explain the 30% reduction in cancers.

**Inflammation and ROS:** Inflammatory processes are an important target of our DSP [22] as chronic inflammation is implicated in aging and age-related diseases including cancer [47–51]. Inflammation can impair antioxidant defenses [49] and induce oxidative stress by activating oxidant-generating enzymes such as NADPH oxidases, inducible nitric oxide synthase, myeloperoxidase, eosinophil peroxidase [47] and 5-lipoxygenase (5-LO) [33]. NADPH oxidase (on macrophages and neutrophils) and 5-LO (in lymphocytes) are activated in response to inflammatory cytokines such as interleukin-1β [52] and particularly generate large amounts of ROS [33]. This can in turn upregulate further production of inflammatory cytokines [50]. The oxidative damage resulting from inflammation-induced ROS can exacerbate mutation rates and facilitate tumorigenesis [47]. Indeed, anti-inflammatory drugs can protect against cancer [53,54]. Hence, the anti-inflammatory agents in our DSP likely contributed to decreased tumorigenesis in p53+/−
mice (Table 2). While inflammatory markers were not assessed here, preliminary findings show that the DSP reduced inflammation-associated symptoms in a lupus mouse model of systemic autoimmunity (unpublished).

**Insulin resistance, growth factors and tumorigenesis:** Insulin resistance commonly develops with age [55,56] and emerges in many age-related conditions [57–60]. Considerable evidence suggests a link between insulin resistance and cancer [60–63]. There are eleven ingredients in our DSP known to promote insulin action and glucose utilization [22]. As compensation for lack of insulin sensitivity, insulin resistant individuals are generally hyperinsulinemic, even in the fasting state [61,64]. Elevated insulin inhibits IGF-binding proteins thereby increasing concentration of free IGF-1 in plasma [61]. IGF-1 is the major tissue growth factor stimulating cellular proliferation and acts permissively to regulate many other growth factors [65]. Elevated activity of IGF-1 is particularly associated with cancer development [61,66,67]. This action involves sustained stimulation of cellular growth cascades [60,62,67] including the GH axis mediator, mTOR [see: Ch. 8]. Recently, drugs enhancing insulin sensitivity (e.g. Metformin) were proposed for cancer therapy [68].

We previously reported that the DSP lowered blood glucose levels [Ch. 5] and improved glucose clearance (Matravadia, unpublished). This may reflect enhanced insulin sensitivity. Considering the relationship between insulin resistance and cancer [61,63] this property of the DSP may additionally contribute to moderation of tumor progression as well as initiation.

We did not quantify IGF-1 as levels of free IGF-1 vary with circadian rhythms [69] making it difficult to obtain an accurate measure in a useful temporal framework. However, growth rate of supplemented p53+/- mice was reduced compared to that of controls (Fig. 4A). We also found that supplemented normal mice had slower growth rates in youth despite normal adult sizes (i.e. entirely resembling p53+/- mice here; see: Fig. 4 and [Ch. 8]). Growth is closely regulated by IGF-1 [70]; therefore, slower growth of supplemented p53+/- mice may indirectly reflect IGF-1 status. Given the role of IGF-1
in carcinogenesis [61,66,67], this presents an additional mechanism of interest for tumor suppression by our DSP.

**Physical activity and cancer risk:** Mechanisms linking physical activity to cancer are complex [71], but research generally confirms beneficial impacts [61,71] with rare exceptions [72,73]. We previously reported that supplemented normal mice remain more active and this is extended into older ages [19]. Levels of spontaneous exercise [Ch. 5] and locomotor intensity [Ch. 4] were also elevated in supplemented mice. Thus, higher physical activity may also independently contribute to reductions in tumor rates by the DSP.

**Multiple tumor burden:** One third of untreated p53+/- mice expressed multiple tumors (usually adenocarcinoma and osteosarcoma) arising from different tissues (Table 2). Conversely, multiple tumor burden affected <10% of supplement mice (p=0.013; Table 2). This further supports that the DSP powerfully impacts tumorigenesis. This is relevant in a therapeutic perspective as effective treatment of multiple tumors is more challenging.

**Metastatic tumors and dietary supplementation:** Most solid primary tumors may be treated surgically [74–76] to obtain complete remission [14]. Conversely, metastases spread to multiple sites, eventually becoming unresectable, and requiring chemo or radiotherapy [77]. Such treatments increase survival, though full remission is uncertain and strong side effects may be induced [14]. High mortality (~90% of cancer deaths [29]) reflects metastases as opposed to primary tumors [77,78]. We found that 17% of untreated p53+/- mice developed tumors of metastatic origin (Table 2). Remarkably, no metastases were detected in any of the 45 supplemented mice examined (p=0.004; Table 2).

Metastases form after the primary tumor has considerably progressed and in humans may occur many years after primary tumor formation [78]. Due to compromised tumor suppression, p53+/- mice express rapid tumor growth and reach endpoint fairly quickly. The moderate incidence of metastasis in our mice may be explained by the narrow window between onset of primary tumor and endpoint euthanasia. Regardless, metastases
undetectable in supplemented mice were confirmed in 17% of untreated animals. It is possible that solitary metastatic cells were present at secondary sites; however, none transformed into clinically detectable masses. This suggests that the DSP considerably inhibits metastasis itself or secondary tumor development. Up to a third of cancer patients exhibit metastatic progression at time of diagnosis [79]. Initiating treatment prior to metastatic progression greatly improves prognosis and survival [29]. Assuming that impacts of DSP translate to humans, forestalling spread of metastatic cells would prolong the diagnostic window when there is greater chance for effective treatments (i.e. primary tumors can be treated without metastatic complications).

**Mechanisms of metastasis:** Metastasis begins with shedding of cells from a primary tumor [78]. Deadhesion is achieved through degradation of extracellular matrix (ECM) proteins by matrix metalloproteinases (MMPs) [77,80,81] which helps release cells into circulation. High MMP activity is associated with metastasis [81] so MMP inhibitors are promising targets for early-stage cancer treatment [82,83]. MMPs also stimulate angiogenesis [80] mediated by the vascular endothelial growth factor (VEGF) [50,84]. High vascularization provides a gateway for cancer cells to enter circulation and local support to rapidly growing tumors [50,77,78]. Subsequently, expansion of blood vessels to sites where metastatic cells arrest is needed to support their metastatic proliferation. Indeed, elevated VEGF exacerbates metastasis [85,86] while suppression of VEGF signaling opposes angiogenesis and metastatic growth [86–88].

VEGF can also increase NF-κB [89] and STAT activation [90]. Survival of cancer cells in circulation is assisted by NF-κB and STAT transcription factor pathways [91]. NF-κB and STAT inhibit apoptosis and thus contribute to survival of cancer cells [91]. NF-κB and STAT inhibitors enhance apoptosis in tumor cell lines [91–93] and demonstrated promising results for cancer therapy [94].

Homing on target tissues is a crucial step in metastatic invasion. Tumor cells can express chemokine receptors complementary to chemokines found on cells of commonly invaded organs [95]. A chemokine-receptor match activates pathways that induce cytoskeletal changes [78]. This increases cell motility [78] and facilitates movement of
cells into the secondary site [96]. Cytoskeletal organization and cell motility are regulated by Rho GTPases [97–99] and their role in cancer metastasis was confirmed [99,100]. Chemokines also induce expression of MMPs [48,50,101] which degrade the ECM allowing better movement of invading cells through the matrix [96].

Once arrested in tissue, the vast majority of the solitary cells will die, remain dormant or form small pre-angiogenic metastases [78]. Only a few in millions of circulating cancer cells will eventually give way to clinically detectable masses [78]. Therefore, at this pivotal stage, survival and proliferation are particularly crucial and require profuse growth factor stimulation. Once again, MMPs play a key role. MMPs can cleave and release growth factors and cytokines tethered to the ECM [77], and activate latent growth factors via proteolytic cleavage [80].

In summary, metastatic development converges on four critical mechanisms: ECM erosion and remodeling, angiogenesis, enhanced cell vitality, and cytoskeletal reorganization. As discussed above, these mechanisms are principally mediated by MMPs, VEGF, NF-κB, STAT and Rho GTPases.

It was shown that ROS stimulate activity of MMPs [50,102], VEGF [103], NF-κB [104] and STAT [105]. Likewise, inflammatory cytokines control MMP expression [106] and stimulate NF-κB, STAT [91] and VEGF [107]. Growth factors (such as IGF-1) induce Rho GTPase activity [108], and expression of VEGF [109] and MMPs [110]. Therefore, targeting oxidants, inflammatory process and growth factor pathways is expected to hinder metastatic development at all key junctures. We argued that our DSP simultaneously modulated oxidative, inflammatory and growth factor levels. Considering the mechanisms responsible for metastasis, our DSP should obstruct metastatic progression. Indeed, this is strongly supported (see: Table 2). As discussed above, forestalling metastasis is critical to improving survival rates.

**Late intervention and age of tumor onset:** Cancer risk increases with age [14]. Indeed, the largest proportion of tumors affected the oldest age cohort of our p53+/- mice (Fig. 1). Furthermore, the greatest impacts of DSP treatment were also observed in oldest mice. Supplementation had no impact on tumorigenesis in younger mice, but
significantly reduced tumor incidence in mice aged over 650 days (Fig. 1). The core of our DSP was formulated to target mechanisms associated with aging [17–20]. The majority of age-related dysregulations are shared with mechanisms of tumor development [2,6,9]; hence we expected to see greater benefits in older animals.

At the same time, older animals obtained prolonged treatment by virtue of living longer. Full-dose supplementation was achieved by ~6 months (180 days) of age. Two supplemented mice reached tumor endpoints between 250 and 350 days of age followed by four, two and three mice succumbing to tumors at each 50 day interval thereafter (Fig. 1). In mice, solid tumors may take up to a month to attain a detectable size [111] and longer to reach endpoint volumes. Therefore, with respect to the youngest mice, tumor development may have commenced before treatment began or shortly thereafter. In either scenario, an abrupt treatment course is unlikely to yield any substantial benefits. This lessens the relevance of data gathered from young mice with respect to our treatment. Conversely, animals supplemented for longer periods represent a more suitable cohort for assessing treatment impacts. Indeed, older supplemented cohorts displayed diminished tumor incidence attesting to the efficacy of our DSP (Fig. 1). Regardless, even with inclusion of young animals, DSP treatment significantly reduced overall tumor incidence (30% difference; Table 2).

Cancers in normal p53+/+ mice, similar to humans, appear primarily in older ages [8]. Thus, greater benefits may be expected in normal phenotypes.

**Longevity:** We previously reported an 11% longevity extension in DSP treated normal C57BL/6J×SJL mice [18]. Surprisingly, analysis of p53+/- survivorship did not detect a significant difference in longevity of supplemented and control mice (Fig. 2) despite a marked reduction in lifetime tumor frequency. This may reflect that there was little difference in tumorigenesis between treatments prior to 600–650 days (i.e. nearly two years of age) with large impacts of DSP treatment emerging only in the oldest ages. In addition, there is some indication that greater longevity of normal mice [reported in: 19] was largely driven by increased lifespan of females while males were somewhat less
affected. All mice used here were males suggesting that impacts of DSP on female p53+/- lifespan may not be ruled out.

CR has the greatest impact on longevity of all known interventions. CR mice with intact p53 expression have nearly 40% longer life [112] and reduced cancer risk [46]. However, calorically restricted p53+/- mice attain longevity extensions of only 8 to 19% [16]. This suggests that impacts on lifespan translate poorly to animals with compromised p53 expression, which may also account for lack of longevity extension at present (Fig. 2). Regulatory involvement of p53 in cell senescence [9] may be implicated. Surprisingly, CR did not reduce tumor incidence in p53+/- mice [16]. This suggests that out DSP appears to be a better intervention compared to CR.

**Exceptional longevity and tumor incidence:** The long-lived Ames dwarf mice deficient in GH attain longevity extension of up to 50% (i.e. doubled lifespan) [113]. However, these mice are similar to CR p53+/- animals with respect to tumor incidence, in that they develop tumors later in life, but tumor incidence is not reduced [16,114]. In fact, male Ames dwarves were 66% more likely to develop various cancers than normally aging mice [114]. It appears that CR or GH deficiency without additional intervention may have poor or no effects on tumor incidence per se. Rather, the delayed onset of tumors may simply reflect that lifespan of these mice is extended over a longer period.

**Longevity and dietary consumption:** Some mice did not consume a full dose of the DSP on a daily basis. Only 41% of mice consistently ingested >95% of their daily doses. Regardless, our delivery mode remains largely adequate since >85% of mice ingested at least 80% of full dose over lifetime. In previous studies full consumption was always achieved [19–21]. The bulkiness of the present supplement compared with a smaller load used in previous studies was likely a factor. Interestingly, maximal longevity was strongly correlated with dietary consumption (p=0.0001; Fig. 3). The longest lived animals showed highest DSP consumption indices (Fig. 3). In fact, excluding mice with lowest consumption indices from longevity curves slightly improves the data (not shown). By the same token, inconsistent consumption could obscure impacts of treatment on tumorigenesis.
Reducing bulk should increase palatability. Tablets and capsules manufactured for human consumption often contain large quantities of non-active ingredients (filler). Using pure ingredients is a better approach in rodent models.

**Body size, growth, dietary supplement and p53:** The p53 pathway negatively impacts growth and proliferation via activation of cell cycle arrest [2]. Mice with amplified $p53$ expression have reduced body weight and diminished tumorigenesis (i.e. body mass is negatively associated with p53 levels) [11,115]. Consistent with this relationship, size range for male C57BL/6 mice\(^{28}\) is normally $<35$ g, but our $p53^{+/-}$ mice attained mature sizes of $>48$ g (Fig. 4). Supplementation resulted in a significant reduction of growth rates (Fig. 4A) which may reflect impacts of DSP on growth factors pathways (see earlier discussion and [Ch. 8]). Contradictory, however, no change in adult body mass between treatments was obtained (Fig. 4B).

Another mechanism may involve direct effects of supplementation on p53 activity. Curcumin and resveratrol (both in our DSP) were shown to increase $p53$ expression and p53 nuclear translocation [116] which is expected to negatively impact aspects of growth [115]. Molecular measurements or cellular p53 dynamics in conjunction with DSP treatment are required for additional certainty.

**Role of p53 in aging:** Involvement of p53 in aging was recently highlighted [6,9,11]. The p53 system responds to a variety of stress signals and DNA damage in particular [6]. Active p53 protein initiates transient cell cycle arrest until stressors diminish and to allow for DNA repair [9]. If stressors persist or DNA repair fails p53 remains active which leads to apoptosis or cell senescence [9]. This ultimately results in compromised tissue renewal, cell depletion and aging [9]. Though exact mechanisms are still debated [6], mice overexpressing p53 exhibit drastic premature aging [11,115]. Likewise, mice with hyperactive p53 protein are smaller and shorter lived, but a normal phenotype is rescued by blocking p53 function [9]. In that respect, $p53^{+/-}$ mice should be longer lived, but due to accumulated mutations and unchecked proliferation of cancer cells their lifespan is actually reduced [9]. It appears that the negative tradeoff between tumor suppression and

\(^{28}\) Jackson Laboratory phenotype information
aging ultimately limits lifespan. In addition, involvement of p53 in age-related neurodegenerative diseases was also highlighted [117].

Given the unfavorable consequences of sustained p53 activation, cells maintain this protein under tight regulatory control [9]. MDM2 inhibits p53 action by binding active domains of p53, facilitating p53 nuclear export and promoting p53 degradation [118]. Loss of \textit{MDM2} is lethal both in embryonic and adult life stages [119–121]. At the same time, moderate reduction in \textit{MDM2} expression expectedly improved tumor suppression but did not reduce longevity [122]. While it remains largely unexplained [6,9] this finding uncovers the possibility of loopholes in the strict tradeoff between aging and tumor suppression. Broad regulatory effects of p53 [6] suggest a variety of downstream effector pathways. By targeting intermediates of these specific pathways it may be possible to selectively balance certain actions mediated by p53 effectively “customizing” the desired outcome. For example, lowering the threshold for activation of DNA repair while elevating the apoptotic threshold may simultaneously improve tumor suppression and prevent cell depletion.

The reduced growth rate of supplemented mice (Fig. 4A) may point to heightened p53. Yet, the fact that adult body mass was not affected (Fig. 4B), and that lifespan was unchanged (Fig 2), argues against impacts of DSP directly on p53 levels. It is more likely that other elements (e.g. redox, insulin sensitivity, growth factors) are contributing. At the same time, it is possible that negative impacts on longevity were counterbalanced by the anti-aging properties of our DSP. In any respect, the double role of p53 (i.e. tumor suppression vs. aging) must be carefully considered in relation to risk factors when exploring treatment options. Use of ingredients that target p53 (such as curcumin and resveratrol) in long-term preventative treatment may require re-evaluation.

**Reformulation of supplement:** The original formula of our DSP [19–21] was upgraded to include more targets specific to tumor development by adding five new ingredients: curcumin, lycopene, pomegranate extract, quercetin and resveratrol. Table 3 lists the biological actions of these agents relevant to mechanisms of cancer gathered from recent literature. In addition, dosages of ginger root extract and ginseng were
slightly decreased only to reduce bulkiness and improve palatability (Table 1). Otherwise, original dosages should be used as given in [19–21].

7.5 ACKNOWLEDGEMENTS

We thank Dr. Douglas R Boreham for generously donating p53+/- mice and Zoya Tov for assisting in preparation of the dietary supplement. This study was supported by a grant to Dr. C David Rollo from the Natural Sciences and Engineering Research Council of Canada.
7.6 FIGURES and TABLES

Table 1. Components and dosages of the DSP

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<th>Component</th>
<th>Dose (mg/day/100 mice)</th>
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<tr>
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<td>30.49</td>
</tr>
<tr>
<td>Lutein</td>
<td>16.59</td>
</tr>
<tr>
<td>Lycopene</td>
<td>8.28 †</td>
</tr>
<tr>
<td>Magnesium</td>
<td>24.67</td>
</tr>
<tr>
<td>Melatonin</td>
<td>0.73</td>
</tr>
<tr>
<td>N-acetyl Cystein</td>
<td>304.88</td>
</tr>
<tr>
<td>Pomegranate Extract</td>
<td>162.89 †</td>
</tr>
<tr>
<td>Potassium</td>
<td>18.11</td>
</tr>
<tr>
<td>Quercetin</td>
<td>621.09 †</td>
</tr>
<tr>
<td>Rutin</td>
<td>74.62</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.05</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>165.62 †</td>
</tr>
</tbody>
</table>

† Newly added ingredients
§ Dosages were cut to reduce bulkiness of supplement
All changes are addressed in discussion
Table 2. The number of p53+- mice developing tumors was 30% fewer in supplemented animals. Types of tumors observed and their frequencies are compared below. Significant probabilities for DSP impacts are bolded.

<table>
<thead>
<tr>
<th>Mice affected by tumors</th>
<th>Untreated</th>
<th>Supplemented</th>
<th>Chi-Square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34/41</td>
<td>26/45</td>
<td>p=0.018</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Observed tumor types</th>
<th>Number</th>
<th>%</th>
<th>Number</th>
<th>%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma</td>
<td>22/41</td>
<td>54%</td>
<td>19/45</td>
<td>42%</td>
<td>p=0.387</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>13/41</td>
<td>32%</td>
<td>8/45</td>
<td>18%</td>
<td>p=0.208</td>
</tr>
<tr>
<td>Soft tissue sarcoma</td>
<td>9/41</td>
<td>11%</td>
<td>9/45</td>
<td>17%</td>
<td>p=0.761</td>
</tr>
<tr>
<td>Astrosarcoma</td>
<td>0/41</td>
<td>0%</td>
<td>1/45</td>
<td>2%</td>
<td>p=1.000</td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>0/41</td>
<td>0%</td>
<td>1/45</td>
<td>2%</td>
<td>p=1.000</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>16/41</td>
<td>39%</td>
<td>6/45</td>
<td>13%</td>
<td>p=0.031</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>14/41</td>
<td>34%</td>
<td>6/45</td>
<td>13%</td>
<td>p=0.039</td>
</tr>
<tr>
<td>Basosquamous carcinoma</td>
<td>2/41</td>
<td>5%</td>
<td>0/45</td>
<td>0%</td>
<td>p=0.224</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>9/41</td>
<td>22%</td>
<td>5/45</td>
<td>11%</td>
<td>p=0.389</td>
</tr>
<tr>
<td>Pheochromocytoma</td>
<td>2/41</td>
<td>5%</td>
<td>0/45</td>
<td>0%</td>
<td>p=0.224</td>
</tr>
<tr>
<td>Multiple tumor burden†</td>
<td>13/41</td>
<td>32%</td>
<td>4/45</td>
<td>9%</td>
<td>p=0.013</td>
</tr>
<tr>
<td>Metastasis</td>
<td>7/41</td>
<td>17%</td>
<td>0/45</td>
<td>0%</td>
<td>p=0.004</td>
</tr>
</tbody>
</table>

†Number of mice diagnosed with two or three different primary types of tumors
Table 3. New components added to DSP and their biological impacts on mechanisms associated with spontaneous tumorigenesis and metastasis

<table>
<thead>
<tr>
<th>Component</th>
<th>Target Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>MMPs and VEGF inhibition</td>
<td>[83,88]</td>
</tr>
<tr>
<td></td>
<td>NF-κB modulation, apoptosis</td>
<td>[88,116]</td>
</tr>
<tr>
<td></td>
<td>Cell cycle arrest</td>
<td>[88,135]</td>
</tr>
<tr>
<td></td>
<td>Redox balance</td>
<td>[88,136]</td>
</tr>
<tr>
<td></td>
<td>p53 tumor suppression activation</td>
<td>[116]</td>
</tr>
<tr>
<td></td>
<td>STAT and growth factor suppression</td>
<td>[88]</td>
</tr>
<tr>
<td>Lycopene</td>
<td>IGF-1 signaling inhibition</td>
<td>[128]</td>
</tr>
<tr>
<td></td>
<td>Redox balance</td>
<td>[35]</td>
</tr>
<tr>
<td></td>
<td>Cell cycle arrest, apoptosis</td>
<td>[127]</td>
</tr>
<tr>
<td>Pomegranate Extract</td>
<td>IGF-1 modulation, apoptosis</td>
<td>[132]</td>
</tr>
<tr>
<td></td>
<td>VEGF inhibition, NF-κB modulation</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>Antioxidant protection</td>
<td>[134]</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Cell cycle arrest</td>
<td>[129]</td>
</tr>
<tr>
<td></td>
<td>MMPs inhibition</td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>Antioxidant protection</td>
<td>[131]</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>STAT3 blocker</td>
<td>[92]</td>
</tr>
<tr>
<td></td>
<td>Antioxidant protection</td>
<td>[125,126]</td>
</tr>
<tr>
<td></td>
<td>p53 tumor suppression activation</td>
<td>[116]</td>
</tr>
<tr>
<td></td>
<td>Apoptosis</td>
<td>[116,123]</td>
</tr>
<tr>
<td></td>
<td>Insulin sensitivity</td>
<td>[125]</td>
</tr>
<tr>
<td></td>
<td>NF-κB modulation</td>
<td>[124]</td>
</tr>
</tbody>
</table>
Figure 1. Cumulative proportion of mice in each treatment group diagnosed with endpoint tumors (and sacrificed) across age intervals. Proportion of mice displaying tumors was nearly identical in younger (<600 days) mice. New tumor cases were rare in supplemented mice older than 600 days but were frequent in age-matched untreated controls. A significant difference in cumulative tumor incidence was resolved by age 650 days ($p < 0.018$).
Figure 2. Survivorship curves for supplemented and untreated p53+/- mice. No significant differences in overall survivorship or maximal longevity were resolved using a log-rank test. Several young mice (<320 days old) culled due to skin lesions with no signs of tumorigenesis were excluded.
Figure 3. Supplemented mice only: Relationship of DSP consumption index to maximal longevity. Higher dietary consumption was strongly correlated with longer lifespan. Linear regression: $y = 0.631 + 0.001 \times x$; $r^2 = 0.240$; $p = 0.0001$
Figure 4. (A) Relationship of body mass to age in supplemented (n=60) and untreated (n=54) p53+/- mice between 90 and 300 days (~3–10 months). Slopes of linear regressions (growth rate) were significantly differentiated with ANCOVA (covariate=age) (homogeneity-of-slopes: \( p<0.002 \)). Supplemented mice showed reduced growth compared to controls in the first 10 months of life. Equations of linear regressions (where y=body mass (g); x=age (days)):
Unt: \( y=27.51+0.071x; r^2=0.53; p<0.0001 \)
Supp: \( y=31.16+0.049x; r^2=0.31; p<0.0001 \)
(B) Body mass reached a plateau at ages >400 days so mean body mass of adult (>400 d old) mice was compared with a t-test between supplemented and untreated animals. No significant difference was found indicating that reduced growth of younger mice did not translate to smaller adult body sizes
7.7 REFERENCES


Dröge W. Free radicals in the physiology control of cell function. *Physiol Rev.* 82: 47–95, 2002


[130] Sung MS, Lee EG, Jeon HS, Chae HJ, Park SJ, Lee YC, Yoo WH. Quercitin inhibits IL-1β-induced proliferation and production of MMPs, COX-2, and PGE2 by rheumatoid synovial fibroblast. *Inflammation.* 35: 1585–1594, 2012


CHAPTER 8

Chapter introduction:

This final chapter discusses the relationship between growth and longevity. Exceptionally long-lived mice (Ames and Snell mutant dwarves) exhibit drastic reductions in growth. Likewise, longevity extension by caloric restriction involves inhibition of growth pathways. In Chapter 5 we suggested possible effects of supplementation on growth signals through modulation of nutrient and energy status indicators (e.g. glucose and white adipose tissue). In addition, small but significant reduction in growth of p53+/- mice attributed to supplementation was documented in the previous chapter.

Our supplement does not specifically target growth, however pathways regulating growth are closely implicated in aging. Previous reports and present findings, however, show no change in adult body sizes of supplemented mice. Regardless, the strong link between growth and aging ultimately demands a thorough discussion of possible impacts of our DSP on growth. In spite of no effects of the DSP on adult size, we felt that investigation of growth rates was necessary. Indeed we found that rates of juvenile growth in mice supplemented from birth were reduced compared to control mice.

These findings are presented in conjunction with a synthetic discussion of growth and stress resistance pathways that oppositely impact aging rates. Cellular bifurcation of growth and stress resistance signal transduction is examined with respect to aging and modulation by our DSP. Circadian patterns of systemic regulatory systems are briefly reviewed. Sleep duration of juvenile mice is examined as both growth and stress resistance are closely associated with sleep.

Finally, improved fecundity of supplemented aging female mice is documented. These findings provide important insights into rates of reproductive aging as affected by complex diets. All organisms show negative tradeoffs between growth/reproduction and longevity. Marked tradeoffs are evident in calorically restricted and other long-lived mice (i.e. longer lifespan and lower fecundity and reproductive output). Remarkably, longevity extension and fecundity improvement is simultaneously achieved by the DSP; albeit life extension is less than for calorically restricted animals. Further longevity extension in our mice was likely limited by costs of upregulated reproduction and other functional improvements.

Note: Citation number [15] refers to Chapter 1; and citation number [16] refers to Chapter 2
CHAPTER 8

TITLE

The Impact of Life-Long Treatment with a Complex Dietary Supplement on Growth, Reproduction and Aging of Mice

AUTHORS

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8.1 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box O transcription factors</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>MAPK/ERK</td>
<td>Mitogen activated protein kinases/extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
</tbody>
</table>

(All other abbreviations are explained in text)

8.2 INTRODUCTION

Interspecifically, bigger animals have longer lifespans [1]. Within species, however, this relationship is reversed [2–4]. An inverse correlation between growth and longevity (in members of same species) was first observed by McCay nearly a century ago [5]; however the idea that growth modulates aging rates was later disfavored [explained in:
2]. Over the past two decades intraspecific relationships between body size and aging were revisited and the notion that within species growth and lifespan are negatively correlated regained validity [2–4,6–11].

Intraspecifically, mammals that grow faster achieve larger sizes at maturity [2,12] so size can be used to infer growth rates. Nonetheless different rates of growth may also converge on a targeted adult size, making mature size a poor biomarker for growth rates [see example in: 12]. Rollo [2] documented the link between growth and aging in mice based on comparisons of maximal body mass across groups. Miller et al [8,9] specifically showed that early life growth trajectories strongly influence lifespan.

The present study describes the effects of a complex dietary supplement (DSP) on juvenile growth rates of normal mice. Our DSP was formulated to target key biological markers implicated in aging [13] and was shown to extend longevity [14] and offset motor and cognitive aging [15,16; Ch. 3,6]. The DSP does not specifically target growth. However, we predicted that growth pathways will be ultimately affected as pathways regulating growth are also implicated in aging (see: Discussion). Previously, we had no anatomical evidence to confirm this, as supplemented adult mice attained normal body size, and juveniles did not display readily discerned differences in growth trajectories. This demanded closer examination.

Similar to previous reports [17; Ch. 7], we found no significant differences in mature body sizes between supplemented and untreated mice. However, here we show that supplemented mice had a modest but significant reduction in growth rate during the first eight weeks of life. Slower early life growth is consistent with slower aging rates [2,8,9]. Our data supports the inverse correlation between growth and aging, even when mice ultimately attain identical mature sizes. However, we do not imply causality of diminished growth on life extension per se. Instead, these findings support the prediction that DSP targets common junctions shared by growth and aging pathways. In addition, these findings present a unique investigative opportunity since reduced juvenile growth is uncoupled from smaller mature size.
In previous works [Ch. 5,7] we briefly compared results obtained with phenotypes of extremely long lived mutant dwarf mice (Ames and Snell dwarves [6,18]) to those of our DSP supplemented animals. This work will expand this comparison because the longevity of dwarf mutants owes mainly to downregulated growth [6,19]. Key differences between the long-lived dwarves and our DSP treated mice emerge with respect to functional aging. Namely, the long-lived dwarves have reduced fecundity [20] which appears to be traded off in favor of longevity. However, we presently show that our DSP treated females had greater fecundity than untreated controls. Additional functional deficits in dwarf mice include reduced physical vigor (i.e. intensity of physical activity) [21]. Once again, we showed that our supplemented mice had increased motor function intensity [Ch. 4] and prolonged duration of spontaneous exercise [Ch. 5]. From a human perspective, the prospect of trading off functionality in favor of longevity extension seems undesirable.

In conjunction with improved fecundity in aging females, supplemented mice also displayed elevated paper shredding and nest building. This reflects maternal behaviors that help assure better litter survival.

In addition, we show that sleep duration in juvenile DSP treated mice was reduced. We previously found this to be true of adult supplemented mice [Ch. 5]. The relevance of sleep duration with respect to endocrine regulation of growth will be addressed.

The bulk of the discussion will explore regulation of growth and stress resistance pathways at the cellular level. In particular, we will discuss the discovery of a switch mechanism in the PI3K signal transduction pathway that bifurcates into antagonistic arms driven by mTOR (growth) and FOXO (stress resistance). This essentially represents the molecular circuitry controlling a prominent life history tradeoff between growth and stress resistance. Life extension and functional benefits attributed to caloric restriction and treatments with rapamycin (a suppressor of mTOR) trace to altered mTOR/FOXO balance. Specifically, stress resistance and anti-aging mechanisms associated with FOXO are upregulated and mechanisms mediating growth, reproduction and faster aging rates are inhibited [22–24]. Some phenotypic similarities between calorically restricted, dwarf
and our DSP treated mice [see: Ch. 5] suggest that our supplement acts on cellular pathways common to dwarfs and caloric restriction.

8.3 METHODS

**Animals and diets:** Breeding and husbandry of random-bred C57BL/6J×SJL mice were previously described [15,17] and all protocols adhered to CCAC guidelines.

**Complex Dietary Supplement:** Our complex dietary supplement (DSP) contained 30 ingredients available from health food stores without prescription. Dosages, preparation, administration and biological effects of ingredients are found in [13,15–17]. Mice were randomly assigned at weaning and for life to either the DSP or untreated control group and selected randomly for various experiments.

**Juvenile growth rate:** Pups were weighed at regular intervals between 2 and 56 days of age. The rate of weight gain during the initial 56 day (8 week) period closely followed a linear relationship. Hence, growth rate is reported as grams of weight gain per day. Supplemented (n=12) and untreated (n=12) male/female pairs aged 6–10 months were allowed to mate. Following birth of the first litter the male was removed from the breeding cage to prevent subsequent impregnation. Hence, only a single litter from each breeding couple was obtained. Since litter size can influence body mass of individual pups, unusually small (<5 pups) or large (>12 pups) litters were excluded from the study. Weaning took place at 21±4 days of age. Pups born to supplemented females began receiving dietary supplementation post weaning. Prior to weaning, only the mothers received supplementation.

**Adult body mass:** We randomly selected 30 supplemented and 30 untreated pups used in the growth rate study and maintained them in our colony until one year of age. Weights of one year old mature adults were collected on three consecutive days and averaged.

**Tibia length:** Length of tibia is reflective of skeletal size and body length in mice [25,26]. Mice were killed, hind limbs were removed and tibiae from both limbs were
surgically extracted. Length of tibiae was measured in supplemented (n=21) and untreated (n=20) adult mice (mean age: 1 year).

**Female fecundity:** Fecundity was investigated in aging females 12–15 months old. A single female was placed in home cage with a single male (~1 y/old) for a total of 11 supplemented and 11 untreated mating pairs (supplemented and untreated females were paired respectively with supplemented and untreated males). Following 90 days male mice were removed from cage and females were monitored for an additional 20 days. We assayed fecundity by recording: a) the number of litters produced over the 110 day period, b) latency to birth of first litter from time of paring, c) size of litters (i.e. number of pups born) and d) proportion of pups surviving in each treatment group.

**Sleep Duration:** Duration of sleep in a 24 hour period was recorded for juvenile supplemented (n=9) and untreated (n=8) mice (ages: 38–48 days). Animals were individually videotaped for 24 hours in home cages (photoperiod: 12h light/12h dark). Sleep duration was visually scored from video recording. Short bouts of immobility (<1min) were not counted toward sleep duration.

**Paper shredding:** Mice were singly placed in a standard cage and given a piece of cardboard of known mass (enrichment toy was removed). Mice naturally shred material to build nests. Over the following days cages were checked frequently for shredding progress. When nearly all of the cardboard was shredded, the remainder was removed, dried and weighted. Shredding rate was determined by dividing the weight difference of the cardboard pieces by the time it took mice to shred them. Shredding rate is reported in grams per day.

**Statistical analyses:** Longitudinal body mass data of pups from 2 to 56 days was approximated by a linear fit for supplemented and untreated groups. Growth rates were expressed as slopes of linear regressions. Slopes were differentiated using ANCOVA (covariate=age). Adult body weight and tibia length were averaged for each treatment group and compared with a t-test. For analysis of female fecundity mean latency to birth of first litter and mean litter sizes were compared between treatments using a t-test.
Likewise, a t-test was applied to differentiate between sleep duration of juvenile mice. All analyses were performed using Statistica® 6.0 software.

8.4 RESULTS

**Growth rate:** We investigated the effect of the DSP on the growth rate of C57BL/6J×SJL normal mice. We obtained litters from 12 supplemented and 12 untreated male/female pairs; however, litters with fewer than five or more than twelve pups were excluded (see: *Methods*). As a result, eleven litters from supplemented parents and nine litters from untreated parents remained (Table 1). Mean number of pups per litter were nearly identical for supplemented and untreated groups (7.5 and 8.1 pups/litter, respectively) (Table 2). To establish the rate of growth, pups were weighed at regular intervals from 2 to 56 days of age. Body mass of all pups weighed at a given age were averaged to obtain a mean body weight specific to that age. Mean weights of supplemented and untreated animals were graphed against age and a linear regression function was inserted across each data set (Fig. 1). The slopes of linear regressions were calculated for each treatment group and represent the rate of growth for that group in grams of body mass gained per day.

Figure 1 compares the growth lines for supplemented versus untreated mice. ANCOVA (covariate=age) found that slopes of linear regressions were statistically different (p<0.03). The growth rate for supplemented mice (0.407g/d) was 13% lower compared to that of untreated mice (0.465g/d) (Fig. 1). At 50 days of age, supplemented pups had on average a 17% smaller body weight compared to untreated control pups. Supplemented pups took 7 days longer to achieve a weight of 20g (Fig. 1). We did not record immediate postnatal body mass; however extrapolation of growth curves to day zero suggests that supplemented pups may have been smaller than controls. This requires specific measurements to confirm.

**Adult body size:** We previously reported that adult weight did not differ between supplemented and untreated mice [17]. We wished to confirm here the previous reported results by examining adult body weights of the same mice used in the growth rate study.
We ran a follow-up study on a subset of 60 mice (mean age: 1 year) and found no significant difference in body weights of adult mice between treatments (Table 1). A 4% reduction in adult weight of supplemented mice was found, however this minor difference was not significantly resolved.

Body mass is a good gauge of body size but differences in body fat between treatments could skew the results. Hence, we also measured tibia length in supplemented (n=21) and untreated (n=20) adult mice (mean age: ~1 year) since tibia length best reflects lean body mass [25,26]. We found that tibia lengths were also identical between treatments suggesting that body composition and size was unchanged between groups (Table 1).

**Female fecundity:** We measured whether fecundity of aging normal females was affected by DSP treatment. We assessed 12–15 month old females, which corresponds to the age of declining fecundity in our mice. Supplemented (n=11) and untreated (n=11) females were paired with adult (<1y/old) sexually active males mice for 90 days. The number of litters produced, latency to birth of first litter, number of pups born (litter size) and proportion of pups surviving were measured.

The average latency from time of paring to birth of first litter was significantly lower in supplemented females compared to untreated controls (36 and 62 days, respectively; p<0.03; 42% difference) (Fig. 2A). All 11 supplemented females produced at least one litter, while only 9 of 11 untreated females gave birth (Table 3). Four supplemented females gave birth to a subsequent litter within the allocated timeframe compared to only one untreated female (Table 3). The only female to give birth to three litters was supplemented (Table 3). The average number of pups per litter was lower for untreated females (4–5 pups) compared to supplemented females (6–7 pups); however, this difference was poorly resolved (p=0.13; Fig. 2B). All pups survived to weaning. We established a significant (p<0.03) correlation between the latency to first birth and the litter size (Fig. 3). Data pooled for both treatment groups showed that females giving birth sooner were also more likely to produce larger litters (Fig. 3). Paternal behavior may be implicated (see: Discussion).
**Sleep duration:** We monitored sleep duration in supplemented (n=9) and untreated (n=8) juvenile mice (age range: 38–48 days) over 24 hours (photoperiod: 12h/12h light/dark). Sleep duration was 1.2 hours lower in supplemented mice compared to controls (10% reduction; t-test: p<0.01; Fig. 4).

**Paper shredding:** Rate of paper shredding was not statistically differentiated between sexes; even though females had a 14% higher rate (p=0.589). Sexes were combined to test for effects of DSP treatment. Supplemented mice (n=20) showed a 90% higher (nearly double) rate of paper shredding compared to untreated controls (n=24). Despite considerable individual variability a significant difference was resolved (t-test: p=0.040; Fig. 5). All mice were ~1 year of age.

8.5 DISCUSSION

We showed that growth rates of pups born to DSP treated females (and subsequently supplemented after weaning) were significantly lower in the first two months of life compared to untreated pups. Differences were not striking, with a peak divergence in body mass of ~17% at 50 days of age. Supplemented mice later caught up reaching comparable adult body sizes to normal controls and identical skeletal proportions. Adult supplemented mice were only 1.6 grams (4%) smaller as adults, and we suspect that this reflected greater physical activity [15; Ch. 5] and associated lower body fat. Skeletal proportions showed less than one percent difference.

Our complex supplement was specifically formulated to target biomarkers of aging with no consideration of growth. Previous reports showed an 11–28% longevity extension [14], and prominent functional, physiological and behavioral improvements [13,15–17; Ch. 3–6]. However, many pathways implicated in aging are ultimately those involved in growth and energy metabolism; thus we suspected that growth may be ‘unintentionally’ effected. Previously, we were unable to document any impacts on growth as adult mice attained identical body mass. At the same time we considered that reduced growth rates, as opposed to final adult size, may anatomically reflect the status of growth (and therefore aging) pathways.
Role of mTOR in growth and aging: A review by Zoncu et al [27] highlights the involvement of the mTOR signaling pathway in onset of cancer, diabetes and aging. Considering that DSP treatment significantly ameliorated cancer rates [Ch. 7], blood glucose [Ch. 5] and longevity [14] it is plausible that these effects were collectively mediated in part via suppression of mTOR activity or at least antagonism to mTOR impacts. In addition, mTOR is activated by ROS [28] and we showed that supplementation reduced markers of ROS damage [15,29]. There may be a threshold aspect here since strong ROS stress can activate stress response systems (i.e. FOXO, antagonistic to mTOR; see below). Regardless the most parsimonious explanation is a slight reduction in mTOR activity in supplemented mice. Finally, the mTOR pathway is a cellular effector of growth hormone signaling and a crucial mediator of growth (via regulation of protein synthesis and cell proliferation) [30]. Inhibition of mTOR can suppress growth of mammalian cells [31]. Hence, reduced growth rates of supplemented mice reported here are once again consistent with the possibility of mTOR downregulation.

Rapamycin: Harrison et al [24] reported that mTOR inhibition by rapamycin achieved a 9%–14% longevity extension in mice which is closely comparable to the 11% longevity extension observed in our DSP mice [14]. Also, rapamycin is known to have tumor-suppressive and immunosuppressive action via downregulation of mTOR [32,33]. Again, we reported that our DSP reduced cancer incidence in p53+/- mice [Ch. 7] and alleviated symptoms of inflammation in murine models of autoimmunity (unpublished). However, supplementation with our DSP provided an array of additional functional benefits in old age not attainable with rapamycin treatment alone. Specifically, we showed that supplementation prevented age-related declines in cognitive and motor function [15–17; Ch. 5,6], boosted mitochondrial function and neurotransmitter expression in brain [15,16,29; Ch. 4], enhanced muscle strength [Ch. 4], augmented behavioral activity and improved sensory functions [Ch. 3]. Presently we also show that the DSP improves fecundity of older females. Hence it is apparent that the benefits of our supplement encompass and extend beyond those of rapamycin therapy. This also indicates that while
DSP treatment may involve modulation of the mTOR pathway, additional (and perhaps greater) effects are propagated via different mechanisms consistent with the multi-targeted approach of our intervention.

**Nutrients and growth factors activate mTOR:** The mTOR protein functions as a nutrient-sensing molecule [32] and is activated in presence of amino acids and growth factors like insulin and IGF-1 [30,32]. Nutrient uptake by the cells is regulated by growth factors via modulation of mTOR [34]. Inhibition of mTOR by withdrawal of growth factors initiates a cascade of events leading to loss of glucose and amino acid transport across cell-surface transporters arresting cellular growth [34]. Nutrient sensing by mTOR also involves AMPK, which downregulates mTOR in response to energy shortage [35]. Collectively, activation of mTOR depends on two elements: abundance of nutrients and stimulation by growth factors. In this capacity, mTOR orchestrates cellular growth and survival to nutrient availability [32,34].

**Nutrient sensing arm of mTOR:** We previously showed that food consumption did not differ between supplemented and control mice [Ch. 5]. However, DSP treated mice had significantly lower blood glucose both in the fasting and post-meal states [Ch. 5]. Roos et al [36] found that blood glucose concentrations were positively correlated with activation of mTOR in a dose response manner. Lower blood glucose levels diminished mTOR signaling and reduced amino acid transport [36]. In this fashion, while food intake was unchanged in our supplemented mice, lower fasting blood glucose is expected to reduce growth via inhibition of mTOR regulated pathways.

**Growth factor activation of mTOR:** In mammals, growth factor mediated activation of mTOR proceeds mainly via the PI3K pathway [30,32]. PI3K is a major growth signal transduction pathway promoting cell growth, proliferation and survival [37]. Inhibition of PI3K/mTOR results in reduced cell size [31] and may eventually lead to induced cell death [34]. Growth hormone, insulin and IGF-1 activate mTOR via PI3K [38,39] and to some extent via MAP-ERK [40]. Activation of PI3K is triggered by tyrosine kinase receptors following growth factor binding [41]. As a result, PI3K activates Akt which in turn phosphorylates mTOR [34,42,43]. Inhibition of mTOR negatively impacts growth
[31], thus slower growth of supplemented mice is consistent with either reduced or curtailed mTOR stimulation.

**Bifurcation of the PI3K pathway:** Akt is the key switch element regulating the bifurcation of the PI3K pathway (mTOR vs. FOXO activities). In absence of PI3K activation, Akt diverts the cellular state from growth to stress resistance via disinhibition of FOXO transcriptional activity in the nucleus [39]. Alternatively, when PI3K is activated, Akt inhibits FOXO activity and promotes its translocation to the cytoplasm where it may be degraded, restoring signal flow to mTOR [39,41]. Interestingly, in its active state, mTOR can impose negative feedback on growth signal transduction directly upstream of PI3K [38]. This regulatory capacity of mTOR constitutes additional peripheral modulation of growth.

**Stress resistance pathways:** Stress resistance is associated with reduced growth in a conserved ecological tradeoff [44,45]. This relationship traces to the bifurcation of PI3K and mTOR/FOXO regulation [39]. When growth factors are present, Akt phosphorylates FOXO triggering translocation of FOXO from the nucleus to the cytoplasm [41]. In absence of growth factors, FOXO relocates to the nucleus and initiates transcription of genes responsible for cell growth arrest and stress resistance [41]. This closely synergizes with mediators of the stress hormone axis (e.g. glucocorticoids) [46]. In this capacity, Akt appears to be a toggle point between cell growth (mTOR) and stress resistance (FOXO). It also follows that mTOR and FOXO occupy antagonistic arms of the PI3K pathway which consequently appear mutually exclusive [39]. In addition to slower growth and increased lifespan of supplemented mice, we showed that the DSP greatly elevated stress resistance [13]. Markers of DNA damage in supplemented mice were two-fold to six-fold lower following a 2-Gy dose of whole body radiation compared to controls [13]. Also, age-associated nitrosative and oxidative damage biomarkers were significantly reduced in DSP mice despite elevated mitochondrial function [15–17]. This suggests that DSP treatment creates a shift away from growth and towards stress resistance. On the cellular level, this may imply modulation of PI3K such that the mTOR/FOXO balance is altered in favor of the latter.
Stress resistance, longevity and endocrine regulation: Longevity is associated with stress resistance and slower growth [39,44,45]. We have described above that reduced growth and elevated stress resistance result from suppression of PI3K. Hence, PI3K downregulation should also extend lifespan. Indeed, the regulatory impacts of PI3K on aging are well established [39,47–49]. Suppression of PI3K elevates stress resistance, reduces tumorigenesis and extends longevity [32,47–49]. As discussed above, these effects can also be achieved by targeting mechanisms downstream of PI3K (e.g. at mTOR and FOXO). However, in accordance with the hierarchy of regulatory cascades in organisms, modulation of downstream targets is mainly propagated from upstream effectors. In specific terms, activity of PI3K is largely dependent on growth factor expression. Growth factors are messengers of the endocrine system and directly regulate PI3K activity (see above). Therefore, growth, stress resistance and longevity assurance ultimately trace back to endocrine regulation. Targets of PI3K are sensitive to nutrient deficiency [35], but under steady state conditions, endocrine control takes central stage. In this capacity, the aging process is effectively driven by growth factors.

Endocrine regulation of aging has been closely examined [2,6,50,51]. Mutations leading to loss of growth factor expression result in stunted growth and prolonged lifespan [6,19,21,25] while growth hormone transgenesis upregulates growth and accelerates aging [7,50,52,53].

Patterns of endocrine growth factor secretion follow a temporal synchronization with circadian rhythms. Growth hormone (GH) is the central regulator of mammalian growth; however, most actions of GH are mediated by IGF-1, which is secreted by peripheral tissues in response to GH [54]. Mechanisms of GH release are linked to clock genes ensuring a timely output of growth factors with respect to sleep-wake cycles [39].

Indeed, the highest burst of GH release consistently occurs shortly after sleep onset [55]. Synchronization of growth with sleep is an important energy allocation strategy [52,56]. Limiting growth-related functions to sleep ensures that energetic costs of growth do not interfere with wakeful behavioral activities essential for competitive niche interactions.
Sleep, waking and endocrine regulation windows: Sleep is not entirely devoted to growth. Rollo [39] argues that late sleep is actually associated with inhibition of growth stimuli and activation of stress resistance pathways. Rising levels of somatostatin and IGF1-binding proteins in late sleep terminate GH release and inhibit IGF-1 activity [39]. This results in downregulation of mTOR and activation of FOXO [39]. Hence sleep appears to be temporally partitioned into a window devoted to anabolism and growth (GH/IGF-1/mTOR) followed by a window involved in recharging, generating energy substrates and retooling mechanisms of stress resistance (FOXO).

We show that DSP supplementation significantly shortened sleep duration in both young (Fig. 4) and adult mice [Ch. 5]. Hence, it follows that supplemented mice may experience a reduction in one or both the mTOR and FOXO windows. However, it is unclear how these pathways may be differentially impacted. The fact that mTOR is associated with GH secretion and slow-wave sleep whereas FOXO activity is associated with increasing REMS provides a possible biomarker of activity for either pathway [39].

In addition, we previously argued [Ch. 5] that reduced sleep in DSP treated mice may not reflect sleep deprivation, but rather a return to optimal levels of sleep. Rollo [39] argued that increased/extended activity of FOXO in late sleep could be a manipulation likely to extend longevity. However, long sleep duration in humans (>8h) is associated with poor health and increased mortality [57]. The evolved system is designed to invest only a limited amount of resources in stress resistance/longevity assurance [58]. In that framework, longer sleep would likely reflect increased stress requiring upregulation, whereas good health would allow investments (and sleep duration) to be reduced.

We previously reported that giant GH transgenic mice sleep nearly 3.5 hours longer compared to normal controls [52]. This supports a direct relationship between elevated GH and increased sleep duration. A review by Van Cauter et al [59] also shows a clear positive correlation between extended sleep duration and increased GH release. Conversely, prolonged periods of waking were associated with reduced amounts of GH secretion [39,59]. These findings suggest that reduced sleep of DSP supplemented mice is expected to diminish duration of growth factor activity. At the same time, this also
suggests that the FOXO-mediated stress resistance window may too be curtailed. Regardless, the sleep reducing effect of our DSP is expected to negatively influence GH/IGF-1 action, which is consistent with slower growth of supplemented mice.

**Long-lived mice and functional aging:** DSP treated mice resemble models of stress resistance (e.g. slower growth, extended lifespan, reduced ROS damage and elevated resistance to oxidative stress) (see above). The best studied murine models of stress resistance are the Ames and Snell dwarf mice which have drastically reduced levels of GH and IGF-1 [6,18,19,21]. These dwarfs attain longevity extension upwards of 50% [18,44] surpassing that achieved by our DSP [14]. However, the exceptionally long lifespan of Ames and Snell dwarfs is coupled to loss of important behavioral and physiological functions. Ames dwarfs appear to be less active than normal controls [21] and, similar to Snell dwarfs, have markedly reduced fecundity [20,44].

Remarkably, our DSP does not impair functionality. In fact, we showed that DSP treated mice displayed higher levels of locomotor activity [15], exercise [Ch. 5] and physical intensity [Ch. 4]. Presently we found that supplementation actually improved fecundity of older females. This was also associated with increased paper shredding to build nests. More paper shredding implies generally elevated physical activity, but more importantly suggests upregulated maternal and paternal behaviors ensuring better litter care and survival. Fecundity will be further addressed in more detail later in discussion.

**Caloric restriction and stress resistance:** Caloric restriction (CR) is the best known intervention to slow aging in a diversity of species [60]. Our DSP treated mice display a variety of phenotypes shared by CR animals. These include: increased longevity, lower oxidative/nytrosative damage, neuronal protection, better cognitive and physical performance, augmented mitochondrial function, lower incidence of tumorigenesis, and increased stress resistance [see comparison in: Ch. 5]. CR restriction was shown to activate FOXO [46,61]. This was mediated by sirtuins, which were shown to deacetylate FOXO transcription factors [23,61]. The role of sirtuins with respect to our DSP was previously described [Ch. 5]. Given that our DSP effectively mimics CR, it is possible
that stress resistance and retarded aging associated with supplementation may involve FOXO upregulation.

**Oxidative stress and FOXO:** On the one hand, the possibility that FOXO is elevated in DSP mice is supported by the status of nutrient markers. Low blood glucose was shown to upregulate sirtuins [62]. We found that DSP treated mice had reduced fasting glucose levels [Ch. 5], and given the effects of sirtuins on FOXO (see above), it follows that FOXO may be upregulated in supplemented mice. However, induction of FOXO mainly proceeds via stress signals [23,63,64]. Oxidative stress is required for activation of FOXO, which in turn stimulates expression of genes involved in cellular protection against ROS [61,63]. We showed that our DSP significantly reduced markers of oxidative and nytrosative stress [15,29]. Given the large number of free-antioxidants in our DSP, it is quite possible that a considerable amount of oxidative species were quenched without resorting to enzyme-mediated action. In accordance with this mechanism, we expect to see lesser sirtuin expression and therefore, a general suppression of FOXO.

Moreover, the antagonistic relationship between FOXO and mTOR (see previous sections) does not automatically imply that withdrawal of growth factor-mediated activation of mTOR via PI3K should necessarily upregulate FOXO activity in absence of stress-activated input from sirtuins. Additional complexity also emerges as highest levels of mTOR and FOXO activity are temporally segregated into separate windows [39]. Ultimately, it remains unclear whether markers of nutrient shortage (e.g. low blood glucose), or withdrawal of oxidative stress take central stage with respect to impacts of the DSP on FOXO.

**Growth factors, PI3K and mTOR/FOXO in youth and in aging:** Regardless of FOXO, slower juvenile growth suggests that impacts of DSP proceed at least in part via downregulation of PI3K/mTOR. Yet it is unclear whether this apparent downregulation applies to older animals. We must consider that growth factors and PI3K/mTOR system play an important role in the aging brain. mTOR is closely implicated in synaptic plasticity and memory function [65]. Hoeffer and Klann [65] present a review of current pharmacological and genetic evidence showing that PI3K/mTOR pathways are critical.
for learning and memory retention. Similarly, the growth factor-mediated PI3K/Akt signal transduction system is instrumental in neuronal survival [66]. In fact disinhibition of FOXO by loss of growth factor stimulation can lead to neuronal death [66,67].

We previously reported that DSP treatment enhanced learning in old mice and opposed loss of neurons in key brain regions [Ch. 3,6]. While synaptic plasticity and neuronal survival are not entirely at the mercy of PI3K and mTOR/FOXO balance, these finding argue against a general shift away from mTOR, at least in the brain. Given the aging phenotypes of supplemented mice [15–17; Ch. 3–7], it is possible that mTOR downregulation (if indeed present) may be limited to younger ages. While the present growth data (Fig. 1; Table 1) are consistent with such scenario, mechanisms for achieving this (in the context of our diet) are completely unclear.

A more plausible and comprehensive model would include simultaneous upregulation of growth pathways and stress resistance pathways. Given that old supplemented mice appear to embody both augmented cognitive function (mTOR arm of PI3K) and superior stress resistance and longevity (FOXO arm of PI3K) it is possible that both arms are upregulated in a mutually non-conflicting manner. The strict temporal differentiation of mTOR and FOXO into separate windows of action allows for such paradigm.

Female fecundity and resource allocation: The relevance of assessing fecundity in the present context relates to the negative tradeoffs between growth/reproduction and longevity [19,50,52,53,56,68]. In the classical view of the theory of allocation [68], highly demanding functions, must be traded off against one another constrained by energy limitation [52,53,56,68]. In mammals, growth and reproduction are not traded off against each other as they represent the same life-history axis, but together these phenotypes are traded off against longevity assurance [52,53]. Indeed, Bartke and Turyn [19] reviewed data on mice selected for different reproductive or growth traits and showed that fecundity and age of sexual maturation are negatively correlated with longevity. The slower growth of our juvenile supplemented mice may suppress immediate reproduction and re-allocate energy into longevity assurance. This should in turn prolong fecundity into older age, but overall reproductive output is expected to
decline [69]. In the previous section we proposed that growth pathways in DSP treated mice do not appear to be hindered in adulthood. Preliminary data (unpublished) suggests that mTOR activity is not reduced in young supplemented adults and older mice. Indeed, we presently show that reproductive output (litter size and inter-delivery interval) is actually higher in DSP treated mice.

**Fertility and caloric restriction:** Johnston et al [70] found that CR mice were able to ‘have it all’ with respect to preserving normal fecundity and still attaining life extension [70]. Authors claim that the ‘having it all’ theory directly challenges the ‘trade-off’ theory, as CR animals are expected to be further limited by energy constraints. However, the longevity assurance phenotype, in the context of optimal lifetime strategies, comes with a programmed upregulation of fecundity, when immediate mating options are available, food is reintroduced and predation risk is withdrawn [69]. The experimental protocol employed by Johnston et al [70] involved mild CR (75% of *ad libitum* for eleven weeks) followed by returning CR females to *at libitum* food ten days prior to paring. According with the previous statement, reduced fecundity is not expected in this case. Also, authors did not attempt to measure total reproductive output (e.g. number of litters produced over lifetime) [69].

Decades ago experimenters realized that reintroduction to *ad libitum* food was needed for CR females to successfully wean the litter [71]. Mice maintained on CR throughout pregnancy and nursing rarely produced viable litters [72,73]. This clearly shows that true caloric restriction regimes impose major limitations on reproduction. Even when CR females were reintroduced to food during the nursing period, the overall number and size of litters was smaller [71]. Despite the fact that CR females remained fertile longer and produced viable litters at ages unattainable by normal-fed controls, maximal reproductive potential was not achieved [71]. Our DSP does not impose restriction of calories as food consumption remains normal [Ch. 5], yet it appears to mimic many of the beneficial effects attributed to CR (see discussion above). Hence we predicted that DSP treated mice may exhibit prolonged period of fertility (similar to CR) but not decrease litter size
or delivery frequency (unlike CR). In fact, assuming that supplemented females may retain fertility into older ages, the overall output may actually increase.

We did not expect our DSP to have a sizable impact on reproductive output of young mice. In part because oxidative stress does not appear to be a physiologic cost of reproduction [74], and also because our DSP was designed to target the aging phenotype, while reproduction is limited to younger ages. Hence, we only examined fecundity of older females (~1y old). Two of the eleven untreated females were apparently infertile at that age, as no litter was produced even through 90 days of paring with proven breeder males. Conversely, all supplemented one-year-old females gave birth to at least one litter, suggesting that the age of fertility was somewhat extended by DSP treatment.

Differences in fecundity were also apparent when looking at the latency to first litter after paring. Supplemented females gave birth 26 days sooner (42% reduction; p<0.05) compared to untreated controls (Fig. 2A). In addition it appears that supplementation may have shortened the inter-delivery latencies. We did not actually compute the inter-delivery latency, as only one untreated female gave birth more than once; however, given that three supplemented mice produced two litters and one mouse produced three litters in the same time frame, suggest that they were able to recover faster.

Maternal and paternal behaviors: Paper shredding and nest-building behavior is greatly correlated with survival and normal development of pups [75]. In some rodents (e.g. rabbits) paper shredding and nest building is purely a maternal phenotype [76]. Shredding material in mice and rats is not unique to pregnant females, and even males will readily participate in shredding and nest construction [75]. Both maternal and paternal input in attending to nest and litter was shown to be strongly correlated with shorter inter-delivery intervals [77]. We found that paper shredding rates were nearly doubled in supplemented mice compared to controls in both sexes. This suggests that more frequent production possible in DSP treated mice may be partially attributed to improved maternal and paternal behaviors.

Collectively, the above findings suggest that DSP treatment improved fecundity in older aged females. In fact, supplemented mice may actually reach a greater maximal
reproductive output over lifetime. It is rather conclusive that CR (unless rescued by reintroduction of food) has drastic effects on reproduction. The exceptional longevity attained by CR or through genetic mutation (Ames and Snell dwarf mice) is ultimately traded off against fecundity. Our DSP appears to achieve some anti-aging effects of CR and may possibly involve downregulation of growth pathways; however, our treatment maximizes all functional benefits, even that of improved fecundity. The latter, along with other functional improvements [15–17; Ch. 3–6], appears to ultimately limit the degree of longevity extension possible with our DSP. As we alluded to here and in previous works, for aging humans, the collective benefits of maintaining a wide range of functional abilities greatly surpasses mere longevity extension.

8.6 ACKNOWLEDGMENTS

This work was supported by a research grant to C David Rollo (NSERC).
8.7 FIGURES and TABLES

Table 1. Average body mass (n=60) and tibia length (n=41) of untreated and supplemented normal adult mice (mean age: ~1y). Supplemented mice had a 4% smaller body mass and 0.6% shorter tibia lengths but these minor differences were not significantly resolved with a t-test

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Supplemented</th>
<th>P-value</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (±SE)</td>
<td>34.1 ± 0.8g</td>
<td>32.5 ± 1.1g</td>
<td>0.28</td>
<td>4.0%</td>
</tr>
<tr>
<td>Tibia length (±SE)</td>
<td>19.58 ± 0.26mm</td>
<td>19.47 ± 0.25mm</td>
<td>0.75</td>
<td>0.6%</td>
</tr>
</tbody>
</table>

Table 2. The number of litters, mean number of pups per litter and total number of pups used in the study of growth rate. Mean number of pups per litter was nearly identical for supplemented and untreated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of litters</th>
<th>Mean number of pups per litter</th>
<th>Total number of pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>9</td>
<td>8.1</td>
<td>73</td>
</tr>
<tr>
<td>Supplemented</td>
<td>11</td>
<td>7.5</td>
<td>83</td>
</tr>
</tbody>
</table>
Table 3. Results of reproductive success study. Each mating pair consisted of 1 male and 1 female mouse where both mice were either untreated or supplemented. Females were between 12 and 15 months old. Mice were paired for a period of 90 days during which the number of litters born to each couple was recorded.

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Supplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of females producing 1 or more litters</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Number of females producing 2 or more litters</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Number of females producing 3 litters</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Combined total number of births</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>

*Since number of mating couples were the same for untreated (n=11) and supplemented (n=11) groups comparison of raw values is valid.*
Figure 1. Relationship between age and body mass (growth curves) of normal untreated (n=73) and supplemented (n=83) pups recorded between 2 and 56 days of age. Each point represents the mean body mass of all pups weighed at a given age. Growth rates are represented by the slopes of linear regressions for each group. Slopes of lines were 0.407 g/day for supplemented pups and 0.465 g/day for untreated pups (13% difference). ANCOVA (covariate=age) confirmed that slopes of lines were significantly different (p<0.03). Supplemented animals had a 17% smaller body mass at age 50 days compared to untreated controls. Supplemented pups required 7 days longer to achieve a mean body mass of 20 g compared to untreated controls.
Figure 2. (A) Average latency to birth of first litter from the time of pairing for untreated (n=11) and supplemented (n=11) male/female mouse couples. Females aged between 12 and 15 months. Supplemented females, on average, gave birth 26 days (42%) sooner compared to untreated females (t-test: *p<0.05). (B) On average, supplemented females had two more pups per litter (~6–7 pups) compared to untreated females (~4–5 pups). However, differences in litter sizes were not significantly resolved between treatment groups (t-test: p=0.13) due to limited samples and high individual variability.
Figure 3. A significant negative relationship (linear regression: $p<0.03$) was observed between the latency to birth of first litter and litter size (number of pups born). Data pooled for both treatment groups (n=20).
Figure 4. Daily sleep duration was measured in untreated and supplemented juvenile mice (ages: 38–43 days; n=17). Supplemented mice slept 10% less compared to untreated controls (1.2 hours less; t-test: \( *p<0.01 \))

Figure 5. Rate of paper shredding was measured in supplemented (n=20) and untreated (n=24) mice (ages: 13–20 months). Sex differences were not resolved, hence genders were combined. Paper shredding was 90% higher in supplemented mice (t-test: \( *p<0.040 \))
8.8 REFERENCES


[31] Fingar DC, Salama S, Tsou C, Harlow E, Bleins J. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes Dev*. 16: 1472–1487, 2002


CONCLUSIONS

Important conclusions pertaining to findings throughout this work are summarized at the end of each chapter. In this manuscript, I presented and discussed extensive experimental evidence that answers to the title of this work – ‘Complex Dietary Interventions to Slow Rates of Aging’.

The definition of ‘aging rate’ encompasses functional aging as well as longevity. The ultimate goal for aging interventions incorporates prolonged lifespan coupled with preservation of functionality. Unless functionality is maintained into later years, plain longevity extension amounts to merely protracted decrepitude. For elderly humans such prospect hardly constitutes the desired improvement in life quality, and by economic measures, may even be viewed as a step back in gerontological research advancement. Prior to commencing my work, longevity extension by our complex dietary supplement in mice had been confirmed along with evidence for amelioration of age-related cognitive decline in growth hormone transgenic mice. The main objectives of my research converged on assessing the state of functional systems in supplemented aging mice and expanding the analysis to normal animals.

Our complex dietary supplement composed of 30 ingredients was designed to ameliorate five key underlying mechanisms implicated in aging: oxidative stress, inflammatory processes, mitochondrial function, insulin resistance and membrane integrity. A multi-targeted approach reflects the complexly integrated multiple processes contributing to aging. Complex dietary formulations allow targeting multiple biomarkers of aging with possibility for profuse synergetic effects unattainable by single of few ingredient supplements. For humans, an oral supplement is the simplest least demanding (and therefore least likely to be abandoned) mode of intervention. All ingredients are available in health food stores without prescription facilitating rapid translation to the general population once benefits and safety are confirmed by human trials.

Throughout this work, extensive behavioral tests were employed to investigate impacts of supplementation on cognitive, sensory and motor function in aging mice. A range of molecular, physiological and anatomical biomarkers implicated in aging were also
assessed. For virtually every parameter examined supplemented old mice displayed a complete preservation of a youthful phenotype or otherwise a marked improvement compared to age-matched control animals. Improvements extended to general motor function, muscle strength, spontaneous exercise, memory, learning, fecundity, visual acuity, olfactory sensitivity, lower blood glucose, reduced oxidative damage, elevated mitochondrial function and cellular energy, boosted neurotransmitters, higher neuronal populations in key brain regions, greater synaptic density, reduced cancer rates and improvements in further important behavioral functions. Collectively, these parameters encompass strong impacts on the great majority of important biomarkers considered ultimate goals for aging interventions.

In addition to general aging, the present findings have sizeable implications for common age-associated human pathologies such as Parkinson’s, Alzheimer’s, Huntington’s, ALS, diabetes, cancer and vision loss. Results provide roof of principle that complex dietary interventions can powerfully ameliorate age-related functional decline and modulate critical processes implicated in aging.