MIDI MOUSE AS A NOVEL MODEL FOR INVESTIGATING IGF-1 IN AGING

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by
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ABSTRACT

Health in late life is the net result of a complex interplay of biological, psychosocial and environmental factors occurring over a lifetime. One factor modulating this interplay is the hormone Insulin-Like Growth Factor-1 (IGF-1). The focus of this dissertation is to examine the physiological status over the lifespan of mice genetically modified to exhibit low levels of IGF-1 throughout their bodies with the goal of evaluating the use of these mice for testing theories of aging. These mice have been termed "midi mice". Midi mice attained a longer median lifespan, defined as the point in time at which 50% of the population had died. Female midi mice (n=26) achieved a median lifespan of 768 d which was longer (p < 0.01) than that attained by control female mice (725 d, n = 22). Male midi mice (n = 22) attained a median lifespan of 778 d, which was longer than the median lifespan attained by male control mice (721 d, n = 30). Male midi mice also showed an increased maximum lifespan (897 d), defined as tenth percentile survivors, compared to male controls (813 d) (p <0.05). There was no difference in maximum lifespan between female midi mice (813 d) and female controls (822 d), p = 0.11.

We found increased weight in male and female controls from 100 d to 495 d of age, while male and female midi mice maintained approximately the same body weight. At 100 d of age, energy expenditure was approximately twice that of 495 d old mice. Control mice did not vary in physical activity by sex or age, in contrast all midi mice showed a significant increase in physical activity with age. Core body temperature increased with age in all groups except male midi mice, which remained lowest throughout life. Diurnal patterns of energy expenditure, physical activity, and core body
temperature were similar across all groups throughout the lifespan although actual values were different.

At 100 d of age, BMD was lower (p<0.0001) in midi mice compared to controls, with no effect between sexes of either genotype (p = 0.321). At 495±5 d, BMD was lower in midi mice (p<0.0001) compared to controls. Sex also influenced BMD (p=0.010), with males of both genotypes exhibiting lower BMD in older animals. All groups maintained lean body mass and fat mass in the region of interest, defined as the area from the cranial portion of the scapulas to the 100 d to 495 d, p <0.05.

Total protein was extracted from liver of mice (n= 36, 9 per sex and genotype). There was no difference in liver total protein content by sex or genotype (p>0.05). All mice expressed similar non-stress levels of liver HSP70 and liver HSP90, both of which are ubiquitously expressed. Following hyperthermia, male midi and male control mice produced the highest levels of liver HSP90, followed by female midis. Female control mice produced the lowest level of liver HSP90 following the hyperthermia stressor. Following hyperthermia, midi mice expressed slightly higher levels of liver HSP70 than controls. Following hyperthermia treatment, liver CRP levels were significantly lower in female midi mice than both male and female control mice. Liver CRP levels were lower in male midi mice than all other groups. Of unique interest, oxidative damage, measured as nmols carbonyls per mg liver protein in post-hyperthermia treatment mice was higher in midi mice compared to control mice (p < 0.02, n = 36, 9 per group). Mouse common cytokines were also examined, however, no differences were found in liver between control and midi mice for IL1A, IL1B, IL2, IL4, IL6, IL10, IL12A, IL13, IL17A, G-CSF,
GM-CSF (n=6 per genotype; 3 males and 3 females per genotype). These experiments provide a broad starting point, showing that the midi mouse model could be useful in healthspan studies involving inflammation, metabolism, osteoporosis, nutrition, exercise, and innumerable others as was the intention of this project. They also suggest increased oxidative damage is not a factor limiting longevity in this model system.
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Chapter 1 INTRODUCTION

Maintenance of health and vitality with advancing age has been the unrealized dream of health practitioners and scientists for hundreds of years. Now however, there are several promising strategies which have been demonstrated to be beneficial in slowing the aging of model organisms such as flies, worms, mice and rats. These strategies suggest that improving the healthspan of aging men and women may be possible in the future, and are in addition to the well-established benefits of sustaining physical activity and social involvement over the lifespan. The key factor which has enabled new insights and progress in understanding mechanisms of aging has been the availability of one reliable method for retarding aging in laboratory rodents. This method is the simple procedure of reducing daily food intake while maintaining adequate levels of vital nutrients: food restriction without malnutrition, i.e. caloric restriction (CR).

A growing body of literature indicates the insulin/Insulin-Like Growth Factor-One (IGF-1) signaling pathway affects lifespan in model organisms including Caenorhabditis elegans [1, 2], Drosophila melanogaster [3], dogs [4], rodents (rats and mice) [5], [6]. It has been shown that inactivation of the IGF-1 gene or of the growth hormone (GH) receptor that lead to IGFl deficiency in these models prolongs life, particularly in females [7]. In C. elegans mutants of daf-2, a homolog of the insulin/IGF1 tyrosine kinase receptor, live three times longer than wild-type animals [2, 8]. Normally, binding of ligand (insulin/IGF-1) to daf-2 initiates activation of dag-16, a regulatory forkhead transcription factor, which regulates targets that promote dauer formation in larvae and stress resistance and longevity in adults [9]. During starvation, deactivation of
*daf*-2 and reduced *dag*-16 activity, dauer formation (state of arrested development), fat accumulation and delayed reproduction result in increased lifespan [7]. Based on genetic evidence, the mutations that affect dauer and lifespan involve a pathway that includes *daf*-2, coding for a homolog of the insulin and IGF-1 receptors; *age*-1, which codes for a homolog of the enzyme PI 3-kinase; and *daf*-16, which codes for a transcription factor in the FOXO family, of which the closest mammalian homologs are FKHR1 (FOXO1), FKRL1 (FOXO3a), and AFX (FOXO4) [10].

In mammals, correlative evidence of studies in various dog breeds suggests that IGF-1 levels influence lifespan. Breeds of large dogs have much higher levels of IGF-1 than breeds of small dogs, while large dogs have a significantly shorter lifespan than small dogs [4]. In rodents, several models of decelerated aging have been developed, with the majority of these mutations affecting IGF-1 levels, such as in the Snell dwarf mouse [11] the Ames dwarf mouse [12], the Laron dwarf mouse [13], and the *lit/lit* mouse [11, 14]. These mice have extended lifespans in relation to wild type mice. The Snell and Ames dwarfs have mutations in the *pit1* and *prop1* genes, which are critical for pituitary development and the activation of the prolactin and growth hormone genes [7]. Snell and Ames dwarfs are affected by hypothyroidism and a severe reduction in growth hormone, prolactin and thyroid stimulating hormone. The mutation responsible for the *lit/lit* phenotype is a single nucleotide change in the growth hormone releasing hormone receptor that leads to a loss of growth hormone release [14]. The Laron mouse phenotype has a high circulating level of growth hormone, however disruption of the growth hormone receptor/growth hormone binding protein gene prevents cellular response to growth hormone [15, 16]. The above animal modes also have reduced insulin and
glucose levels together with many other changes. A recent review also indicated that some models have an increased or decreased stress resistance [17]. See Table 1. Growth hormone is the primary regulator of IGF-1 in mammals, so all of the above models show a decreased level of plasma IGF-1 in comparison to wild type mice therefore suggesting a relationship between lower IGF-1 levels and increased lifespan.

**Table 1.** Phenotypic characteristics of GH/IGF-1 long-living mutant mice.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>AMES (prop-1)</th>
<th>SNELL (pit-1)</th>
<th>GHR/BP KO</th>
<th>IGF-1R +/-</th>
<th>LID (Liver IGF1 mutant)</th>
<th>Little (GHRHR mutant)</th>
<th>Klotho</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH/IGF1/Insulin signaling</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>Body size</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>⇔</td>
<td>▼</td>
<td>⇔</td>
</tr>
<tr>
<td>Reproduction</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>⇔</td>
<td>⇔</td>
<td>▼</td>
</tr>
<tr>
<td>Insulin sensitivity</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
<td>Partially IGF1 resistant</td>
<td>Insulin resistant</td>
<td>NK</td>
</tr>
<tr>
<td>Stress resistance</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
<td>NK</td>
<td>NK</td>
<td>▲</td>
</tr>
<tr>
<td>Longevity</td>
<td>49-68%</td>
<td>42%</td>
<td>21-40%</td>
<td>33% females</td>
<td>23-25%</td>
<td>18-30%</td>
<td></td>
</tr>
</tbody>
</table>

NK: not known.

Additional evidence suggesting that IGF-1 levels modulate longevity come from caloric restriction studies. The phenomenon was first described by McCay and colleagues at Cornell University in 1935 [18]. The goal of their project was to determine the amount of food necessary to maintain different levels of stable body weight, but the investigators found to their surprise that rats eating less food lived considerably longer than those feeding ad libitum. Much painstaking research over the past decades has demonstrated this effect is broadly based in many different species such as yeast, spiders, fruit flies, fish, mice and rats [19]. In addition, detailed examination of the effect in male Fischer 344 rats revealed that the critical nutritional deprivation required to produce retardation of aging in these animals is the decrease of daily caloric input [20]. Such studies led to the general adoption of the phrase “calorie restriction” or CR, to describe the effect.

Strong genetic dependence for lifespan effects of CR has recently been demonstrated as just as many strains of inbred mice exhibited decreased longevity as exhibited increased longevity in response to 40% CR [21]. This result was obtained in both male and female mice. Caution therefore needs to be exercised in the application of CR to outbred populations such as humans, since the available evidence suggests that genetic background is important for determining both beneficial and adverse effects. Extensive rodent studies have demonstrated the beneficial outcomes of CR to be dependent on the level of restriction and the length of restriction, with greater benefits accruing from higher levels of restriction, in the range of 10-50% less food than ad libitum feeding, and longer restriction times [19, 22]. Beneficial effects are many and varied, such as: increased physical activity over the lifespan, decreased levels of oxidative stress, decreased plasma levels of cholesterol, free fatty acids, glucose, insulin,
the active form of thyroid hormone (tri-iodothyronine), insulin-like growth factor 1 (IGF-1), body fat, increased insulin sensitivity, extended longevity and, notably, elimination or delayed incidence of a wide range of tissue pathology [23-25]. However, as previously mentioned, just as many strains of mice exhibited decreased longevity in response to CR, which may be due to negative effects of CR which have been identified in some studies. Caveats of long term CR identified in rodent studies are: some immune functions are depressed leading to possible increased susceptibility to infectious disease; the rate of wound healing is decreased, reproductive functions are depressed, body temperature is lowered (smaller animals exhibiting more depression of diurnal temperatures) and finally, body size and organ sizes are reduced, with the notable exceptions of the brain (which remains the same size despite reduced food intake) and, counterintuitively, the stomach which increases in size [20, 26, 27].

The changes in physiological characteristics associated with initiation of the CR regimen demonstrate that this simple experimental procedure alters the entire metabolic characteristics of the organism and that this occurs as a consequence of eating the same food but in smaller quantities. It should be noted also that the daily metabolic rate (MR) is reduced immediately following the imposition of restriction [5]. However, long-term CR has been found to be associated with no change in MR, decreased MR or increased MR, indicating that altered MR is not an important component of the beneficial actions of long-term CR [28]. This nutritional regimen is thus associated with both significant beneficial effects for healthspan as well as some adverse effects, and both of these changes are dependent upon genetic background. Despite these limitations the high value of CR lies in the demonstration that longevity and health can be manipulated by
environmental adjustments. Caloric restriction is known to affect metabolism in mice and humans [20, 26]. It is the only proven method to retard aging and has been shown to significantly extend the lifespan of rodents and other animals [19]. One effect of caloric restriction is to reduce IGF-1 levels, which can be significant (up to 50% reduction in plasma IGF-1 levels following a 40% reduction in caloric intake). It has also been suggested the reduced IGF-1 levels mediate the reduction of tumor formation in rats and mice undergoing caloric restriction. Caloric restriction reduces tumor formation in p53 / mice whereas the administration of IGF-1 increases tumor formation and progression to control levels [29]. Given the large amount of correlative evidence of reduced IGF-1 levels with increased longevity, it is important to design studies that will further explore the role of IGF-1 in affecting physiological systems which are known to be important in the maintenance of health and longevity. This is important since studies in lower organisms have shown that manipulation of single genes can affect longevity and health of those organisms. Such genetic manipulations are not possible in free living men and women. However, both genetic and CR studies demonstrate the possible mutability of healthspan and longevity, provided the underlying mechanisms can be identified.
Chapter 2 BACKGROUND AND SIGNIFICANCE OF THE PROBLEM

In mammals, IGF-1 is a pleiotropic growth signal produced primarily by the liver in response to GH. IGF-1 is essential to postnatal growth [30], therefore it is not surprising that complete IGF-1 knockout mice have not been viable strains [30, 31]. Several mouse models of decelerated aging have been developed, with the majority of these mutations affecting IGF-1 levels, such as in the Snell dwarf mouse [11], the Ames dwarf mouse [12], the Laron dwarf mouse [13], and the lit/lit mouse [11, 14]. These mice have extended lifespan in relation to wild type mice. The Snell and Ames dwarfs have mutations in the pit1 and prop1 genes, which are critical for pituitary development and the activation of the prolactin and growth hormone genes [7]. Snell and Ames dwarfs are affected by hypothyroidism and a severe reduction in growth hormone, prolactin and thyroid stimulating hormone. The mutation responsible for the lit/lit phenotype is a single nucleotide change in the growth hormone releasing hormone receptor that leads to a loss of growth hormone release [14]. The Laron mouse phenotype has a high circulating level of growth hormone, however disruption of the growth hormone receptor/growth hormone binding protein gene prevents cellular response to growth hormone [16, 32]. The above animal models have reduced insulin and glucose levels together with many other physiological changes. Growth hormone is the primary regulator of IGF-1 in mammals, so all of the above models show a decreased level of plasma IGF-1 in comparison to wild type mice, therefore suggesting a relationship between lower IGF-1 levels and increased healthspan as was seen in the dwarf rat study [33] and potentially lifespan as was seen in the mouse models. The midi mouse model is an innovative approach to researching problems of aging due to intact GH production, GH and IGF-1 receptors and whole body
reduction in IGF-1 rather than simply reduced plasma IGF-1. This is the first mouse model to allow separation between IGF-1 and GH as modulators of longevity.

Decreased IGF-1 protects mice from the age-associated proteotoxic effects of aggregation of the Alzheimer's disease-linked human peptide Abeta (Aβ) [34]. Life-long reduction of GH/IGF-1 activity was found to attenuate renal damage in rats [35], however conflicting information showed that IGF-1 therapy was effective in improving glucose and lipid metabolism, increasing testosterone levels and serum total antioxidant capacity and reducing caspase activation, and therefore preventing apoptosis, in the rat [36, 37]. In 2005, a study using dwarf rats with a 40% reduction in both GH and IGF-1 showed reduced neoplastic disease, nephropathy, and total disease burden compared to controls, but failed to show any difference in lifespan [33]. Measurement of longevity in days (d) during the first experiment will allow us to determine effects of life-long decreased IGF-1 level on lifespan. Secondly, metabolic rate, physical activity, and core body temperature will be concurrently measured at multiple time points. Such procedures have been performed previously in this lab [5]. Reduced core body temperature may correspond to increased longevity as has been previously shown in mice [38]. The central hypothesis is that lifelong decreased IGF-1 promotes a decreased rate of aging, in part through an enhanced resistance to stress.

Heat shock proteins have a pivotal role in regulating the life and death of cells. Two heat shock proteins of interest Hsp70 and Hsp90 will be examined as part of the animals' stress resistance following a thermal stressor of 42°C for 30 minutes (min) or until CBT reaches 42°C. Heat shock protein 70 supports the folding of newly formed
polypeptides, and also promotes cell survival by inhibiting lysosomal membrane permeabilization in both human and murine fibroblast cell lines [39]. Recently research conducted using osteosarcoma cells showed Hsp70 stabilizes lysosomes by high affinity binding to an endolysosomal anionic phospholipid bis(monoacylglycerol)phosphate (BMP) in acidic environments, such as occurs when lysosomal integrity is breached [40, 41]. This facilitates the BMP binding and activity of acid sphingomyelinase (ASM) [41]. This opens doorways to treatment of certain lysosomal storage diseases such as Niemann-Pick disease [41], and "also reveals a potential strategy for treating cancer by inhibiting the lysosome-stabilizing effects of Hsp70 in tumour cells, thereby promoting lysosome-dependent autophagic cell death, in which the cell digests itself. Autophagy has emerged as a key process that is deregulated during carcinogenesis. So molecules that either inhibit Hsp70-related signaling cascades (such as the PI3K/Akt/GSK pathway, which is linked to up-regulated Hsp70 transcription in cancers), or agents that directly block lysosomal localization of Hsp70, might prove useful in anticancer therapy" [40].

Another recent study using mice null for heat shock factor 1, a transcription factor of hsp s, showed increased Hsp70 expression inhibits pro-inflammatory cytokine production [42]. High Hsp90 expression, the final heat shock protein to be examined in this experiment, was recently shown to be related to longer patient survival in cases of gastric cancer [43]. Up-regulation or down-regulation of heat shock proteins in tumors or cancer may be due to tumor microenvironment and histological characteristics of the tumor type, as up-regulation of Hsp90 expression in breast cancer has been linked to poor prognoses [43, 44]. Previous research in Snell and Ames mice did not follow expectations of increase Hsp production in some tissues measured, possibly due to more
complex mechanisms than exist in long-living daf-2 *C. elegans* mutants [45]. Additionally, both Snell and Ames models have decreased GH and IGF-1 among other hormone alterations previously mentioned, which could have contributed to unexpected results. Using the midi mouse model will allow examination of stress resistance and other physiological factors without the hormonal disturbances noted in the other mouse models.

**Preliminary Evidence**

Male and female midi and control mice (n=106) began the study at 365 days (d) of age. Expected overall survival less than 10% by June 2010. Partial survival analysis by the LIFETEST procedure of SAS indicates that the 50% of the colony is expected to survive to 761.5±9 d. The 95% confidence interval is 729 d to 781 d.

Young animals less than six weeks of age (n = 98; 31 female control, 23 male control, 21 female midi, 23 male midi) were obtained from Dr. Christian Sell's colony at Drexel University College of Medicine, and 30 animals were implanted with G2 e-mitters by S. Motch while still in quarantine facilities as described in the Methods section. Special permission and quarantine area access was obtained from The Pennsylvania State University facilities veterinarian Dr. J. Dodds. As can be seen in Table 2, 24 hr concurrently measured core body temperature (ºC) (n=30), physical activity as measured in distance traveled in centimeters, and metabolic rate at ages 100 d and 180 d shows interesting trends. Hour 1 begins at 0800. Row A depicts animals at age 100 d and row B depicts animals at 180 d. Diurnal variances in temperature and physical activity clearly coincide. As determined by two-factor (sex, genotype) analysis of variance (ANOVA) (model p <0.001) using SAS v. 9.1.3, midi mice showed decreased physical activity.
(p<0.001) compared to controls at 100 d and 180 d of age regardless of sex (p=0.9267). However, female midi mice showed an increased metabolic rate (p<0.001) at both 100 d and 180 d compared to male midi mice and male and female control mice, which could not have been due to higher physical activity. There was an overall decline in metabolic rate and physical activity among all mice between 100 d and 180 d (p<0.001). The preliminary data related to core body temperature and physical activity are in marked disagreement with the decreased rate of aging of calorie restricted mice. These data therefore suggest that lowered tissue IGF-1 may not be an important factor in aging.
Table 2. Concurrent physiological measurements in 100 d and 180 d mice. Hourly core body temperature, distance traveled, and metabolic rate over 24 hr periods in male and female midi and control mice at 100 d (Row A) and 180 d (Row B) of age show declining metabolic rate and physical activity in all mice, with female midi mice having the highest metabolic rate at both 100 and 180 d of age.
Chapter 3 SPECIFIC AIMS AND CENTRAL HYPOTHESIS

The long term goal of these studies is to provide an overview of the midi (IGF-1 hypomorph) mouse model as a research tool, allowing us to better understand how decreased levels of IGF-1 affect lifespan and healthspan, metabolic parameters, body composition, bone mineral density, and response to an acute stress. The central hypothesis is that lifelong decreased IGF-1 promotes a decreased rate of aging, in part through an enhanced resistance to stress. This hypothesis was formulated in part based on animal studies [8, 34, 46-48], recent human studies [49, 50], and preliminary data collected by our laboratory and that of C. Sell (unpublished data). In order to test this hypothesis, the following parameters were used in the midi mouse model as markers of aging over the lifespan: longevity, body composition measures, metabolites, functional measures, metabolic measures and stress response. All procedures received IACUC approval from The Pennsylvania State University. The hypothesis was tested by executing the following Specific Aims:

**Specific Aim #1:** To measure survival characteristics of male and female mice having variable levels of tissue IGF-1 under standard laboratory conditions. Midi mice are expected to have an increased lifespan, in accordance with preliminary evidence shown in the laboratories of our colleagues. The outcome of this aim will evaluate if decreased rate of aging is associated with lowered IGF-1.

**Specific Aim #2:** To measure metabolic characteristics of male and female mice having different levels of tissue IGF-1. Plasma parameters measured over the lifespan via enzyme-linked immunosorbant assay (ELISA) included glucose and insulin. Metabolic
characteristics measured included daily metabolic rate, physical activity, levels of plasma hormones and metabolites. It is expected that animals with decreased IGF-1 will have increased physical activity and metabolic rate and decreased core body temperature. These parameters have been previously shown to be altered in models of reduced rate of aging. Measurements obtained simultaneously over 24 hours (hrs) will be used to assess whole animal metabolic rate (MR), physical activity (PA), and core body temperature (CBT) of both young (100 – 180 d of age) and old (365 – 840 d of age) mice.

**Specific Aim #3:** To measure body composition and bone mineral density in male and female mice having variable levels of tissue IGF-1. In these studies, we will examine the prediction that midi mice will maintain a youthful body composition profile (i.e. more lean mass and less adipose as a percentage of body weight) than controls, although due to lowered IGF-1 levels, it is expected that midi mice may have a lower bone mineral density. Magnetic Resonance Imaging (MRI) and Dual Energy X-Ray Absorptiometry (DEXA) scanning will be used to determine fat and lean masses. Additionally, bone mineral density will be determined via DEXA and body water content will be determined via MRI. The rationale is that these parameters have been shown to change with age.

**Specific Aim #4:** To measure response to acute thermal stress in male and female mice having different levels of tissue IGF-1. Acute stress response will be induced via whole body hyperthermia (WBH). Concurrent measurements of metabolic rate, core body temperature, and behavior will be obtained. Following WBH, mice will be immediately decapitated, whole body blood collected, and tissues (brain, pituitary, liver, heart, spleen, kidney, white adipose tissue, skeletal muscle, femur bone) collected and weighed. Plasma
parameters to be measured via enzyme-linked immunosorbant assay (ELISA) include IGF-1, glucose, insulin, and c-reactive protein (CRP). Tissue parameters to be measured via ELISA include HSP70 and HSP90.

The proposed work is innovative because it capitalizes on a model that has a reduction in IGF-1 throughout the body due to an insertion in the igf-1 gene (Igf1tm2Ts), leading to reduced IGF-1 levels, 30% that of controls [31], and a dwarf phenotype in mice homozygous for the Igf1tm2Ts allele, known as Igf1tm2Ts/tm2Ts, Igf1m/m, or midi mice. Midi mice have high serum levels of growth hormone (GH) and an intact GH response [31]. This finding is important as increased expression of GH has been reported to decrease lifespan in rodents [51]. At the completion of this project, it is expected that outcomes of the above aims will establish the midi mouse as an important and useful model for aging research and possibly for use in other areas of physiological research as well. In addition, these studies will test the involvement of tissue IGF-1 in aging and healthspan.
Chapter 4 METHODS

4.1 Animal and Care Plan

The animal model for all experiments is the midi mouse (IGF-1\textsuperscript{m/m}), created by Lembo and colleagues at the University of California, San Diego in 1996 to circumvent the embryonic lethality of complete IGF-1 deficiency [31]. A brief background of the creation of the colony follows. These mice have IGF-1 levels 30\% of wild type yet survive to adulthood and reproduce, allowing for maintenance of colonies and examination of the phenotype [31]. The F1 heterozygous animals were 129/CD-1 X C57BL/6J, which were then intercrossed to create F2 homozygous midi animals and F2 wild type controls [31]. Midi mice have slightly higher plasma insulin and significantly lower glucose levels than controls and a body size 60-70\% that of controls [31]. The animals used in this experiment are descendents of the colony bred from these animals. Two groups of mice of this genetic descent were obtained from Dr. Christian Sell, Drexel University College of Medicine, Philadelphia, PA for the experiments explained in this document. All experiments were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University. The first group consisted of 106 mice, age 365 d. The second group consisted of 78 weanling and 3 pregnant mice (2 control, 1 midi). All mice were initially quarantined in the Centralized Biological Laboratory (CBL) prior to housing in Research Building D, where they remained throughout testing. The animal room in which the animals were housed was maintained on a 12 hr light cycle with lights on at 0600. Animal rooms were maintained at 23 C and 45 \% humidity. Mice were housed four per cage initially, with no replacement due to attrition, with access to
standard rodent chow (Lab Diet #5001, PMI Nutrition, Intl., Gibsonia, PA) and fresh water ad libitum. Males were separated into groups of two if fighting occurred. Cages were changed at least once per week. Animals were allowed to acclimate to their environment for at least 2 weeks prior to surgery.

Power analysis performed using SAS v. 9.1.3 PROC POWER indicated \( n = 9 \) per group (by sex and genotype) would give the longevity study 0.80 power with 50% survival expected at 24 mo of age. Our total \( n \) was larger to increase the power over 0.90. Similarly, the cross-sectional studies using younger mice required an \( n = 9 \) per group to give 0.80 power. In our studies, we had 9-12 mice per group (by sex and genotype).

Median lifespan of a group was defined as the number of days at which fifty percent of a group had died. Maximum lifespan was defined as the age in days at which 90% of mice of a particular group had died. Each group was defined by sex (male or female) and genotype (midi or control).

4.3 Surgical Implantation of Telemetric Devices

Standard rodent surgical procedures were used. Prior to surgery, the abdomen was shaved using an electric shaver. The area was then cleaned using a povidone iodine surgical scrub followed by ethyl alcohol three times. Surgery involved the implantation of a TA e-mitter (MiniMitter, Respironics, Inc., Bend, OR) into the intraperitoneal cavity in twenty of the first group of mice under isofluorane gas anesthesia using a down-draft table following quarantine and after acclimating to Research Building D housing. The dimensions of the TA emitter are 23 mm x 8 mm with a weight of 1.6 grams (g). Thirty of the younger group of animals were implanted at approximately 8 weeks of age during
quarantine in CBL and were implanted using the smaller and lighter G2 e-mitter (MiniMitter, Respironics, Inc.), which was especially designed for mice, measuring 15.5 mm x 6.5 mm with a weight of 1.1 g. These battery free implantable telemetry devices allow for continuous core body temperature measurement and are energized using the ER-4000 energizer/receiver (MiniMitter, Respironics, Inc.). Pain relief was provided by dripping bupivicaine onto the sutured abdominal muscle wall prior to closing with surgical staples. Absorbable chromic gut suture, size 6-0, was used (Ethicon, Inc., #1641G, Somerville, NJ) for abdominal sutures; Reflex wound clips (7 mm) and applicator were used for wound closure (Roboz Surgical Instruments, Inc., Gaithersburg, MD). Hypothermia was prevented using warmed gel packs during surgery and circulating hot water pads beneath clean cages during recovery. Animals were allowed a minimum of 2 weeks recovery before initiation of experimentation.

4.4 Vaginal Swab of Female Mice to Determine Stage of Estrus

Female mice between 12 and 52 wks of age were examined for signs of estrus prior to testing for metabolic rate, physical activity and core body temperature. It is critical that we know whether or not the mouse was in estrus during the testing of these parameters. Wet-mount smears were immediately viewed via a light microscope and the cellular contents examined to determine the stage of the estrus cycle according to the criteria of Caligioni (2009) [52]. Briefly, a vaginal smear was obtained using a small plastic pipette. The mouse was restrained with one hand and the vulva cleaned with a swab dipped in 0.9% sterile saline (0.15 M) solution. The tip of the pipette was inserted 5 mm into the vaginal opening and the vagina gently flushed with 10 μl sterile saline 5
times with the final flush collected into the pipette. The unstained cells in solution were examined under a light microscope at 10X, 40X or 60X to visualize the cells and determine the proportion of cell types in the flush. Female mice were judged as exhibiting estrus if at least 50% of cells were cornified squamous epithelial cells, which occur in clusters and exhibit no visible nuclei, granular cytoplasm, and irregular shapes. This routine mouse husbandry procedure did not require anesthesia or analgesia. Mice determined to be in estrus were not used that day.

4.5 Measurement of Core Body Temperature

Core body temperature was measured telemetrically each minute with no impediment to the activity of the mouse as it moved about the cage. The TA and G2 e-mitters implanted in the mice are battery-free devices powered by radio waves produced by magnetic coils in the ER-4000 Energizer/Receiver unit. When an animal with an implanted e-mitter is placed in a cage over the ER-4000 unit, the emitter emits a frequency oscillation signal dependent upon temperature. This signal is sensed by the ER-4000 unit and core body is then recorded by software (VitalView Data Acquisition System, MiniMitter, Respironics, Inc., Bend, OR) on a PC independent of other computers mentioned elsewhere.

4.6 Physical Activity

Physical activity was monitored concurrently with core body temperature and measurements for determination of metabolic rate over a period of 24 hrs using an interlocking grid of infrared laser beams projected through the clear 20.32 cm³ metabolic
cage. Each time the animal breaks the path of the beams, movement is recorded, with
distance of movement being recorded in cm. The Columbus Instruments ATM3-Module
OPTO-VARIMEX System (Columbus, OH) provided the infrared laser beam grid, and
was used in conjunction with the Auto Track System 4.0 (Columbus Instruments) on an
independent PC.

4.7 Determination of Metabolic Rate

Oxygen (O\textsubscript{2}) consumption and carbon dioxide production (CO\textsubscript{2}) were measured
concurrently with core body temperature and physical activity. Metabolic rate was
determined via indirect calorimetry using a system designed by Dr. McCarter's
laboratory. This system has been described in detail elsewhere [5]. Briefly, fresh air flow
rate through the cage was held at 500 ml/min (Applied Electrochemistry Technologies,
Pittsburgh, PA, Model R-2), with 150 ml/min (TSI, Inc., Shoreview, MN) of air leaving
the cage passing through a desiccant/membrane dryer (Model 4140, Perma Pure LLC.,
Toms River, NJ) prior to entering gas analyzers. Gas composition was analyzed by a
zirconia cell oxygen detector (Applied Electrochemistry Technologies, S3 A/II) and an
Readings were taken every minute and recorded using MARS software (Applied
Electrochemistry Technologies) on an independent PC. Energy expenditure was then
calculated using the equations of Consolazio et al [53].

4.8 Body Composition Analysis
Analysis of body composition was obtained using DEXA and qMRI technologies. An EchoMRI100 (Echo Medical Systems LLC., Houston, TX) Mouse qMRI machine was used to perform whole body composition analysis without anesthesia as per manufacturer's recommendations. The animal is restrained in a cylindrical plastic tube with adequate ventilation, with the procedure taking less than one minute per mouse. The quantitative magnetic resonance imaging technology has improved precision over DEXA for measurement of total body fat, lean mass, free water, and total body water [54, 55]. A GE Lunar PIXImus 2 (GE Healthcare, Madison, WI) was used to obtain region of interest body composition (from cephalic ridge of scapula to second vertebrate past the pelvis including limbs) and bone mineral density under anesthesia using a mix of xylaxine and ketamine. DEXA technology exposes the mouse to high and low energy x-rays simultaneously, which allows distinguishing of bone, lean tissue, and fat as was previously described [56, 57]. DEXA scanning takes approximately 3 min per mouse, not including time for anesthetizing and recovery. Mice were kept warm in home cages under heat lamps post scanning. Only mice without implanted e-mitters were tested with qMRI or DEXA.

4.9 Tail Vein Phlebotomy

At designated ages, randomly selected sex and genotype matched mice underwent tail vein phlebotomy. Briefly, mice were weighed and restrained in a plastic rodent restrainer. The underside of the tail was nicked with a clean, sterile surgical blade approximate 1.25 cm from the tail base, and blood drops collected into an appropriate EDTA coated glass or polypropylene tube. Less than 1% of the animal's body weight was
taken at one time. EDTA was expected to be approximately 10% of blood collection volume. For example, no more than 300 μl of blood may be taken from a 30 g mouse. Normally, clotting occurred spontaneously, otherwise pressure and a small amount of liquid bandage was applied before placing the animal back into its home cage. Animals are examined 5 min after bleeding to assure clotting and wound sealing. Mice were given a minimum of 2 weeks recovery prior to participating in another portion of the experiment post-bleed. Following collection, blood was placed on ice and immediately centrifuged at 4°C at 2200 x g for 10 min. Plasma was then collected and aliquoted into fresh tubes and stored at -80°C until further analysis.

4.10 Acute Thermal Stress

Acute thermal stress was performed on a subset (n=36) of adult animals only (age = 180±10 d). A large incubator set to 42°C houses the metabolic chamber with forced fresh air circulation (as during other metabolic rate readings) of 500 ml/min maintained through the chamber. Water and rodent chow (LabDiet 5001) were provided ad lib. Oxygen consumption, carbon dioxide production and core body temperature (in animals implanted with e-mitters) are recorded as described above. All animals were observed continuously to ensure well-being, with specific one-minute behavior recordings, and every five minutes noting physical activity, grooming, feeding, drinking, and other behaviors. Animals were maintained in the 42°C environment for 30 min or until core body temperature reached 42°C. At this point, or if an animal exhibited obvious severe signs of stress (excessive salivation, seizure, loss of consciousness, prostrations) it was removed from the hyperthermic environment and sacrificed immediately via decapitation.
4.11 Decapitation

Immediately following acute thermal stress, animals were decapitated via guillotine. Animals were weighed prior to decapitation. Decapicones were used to properly position the animal and ensure safety of the guillotine operator. Trunk blood was collected into an EDTA coated tube and plasma collected as previously described. Tissues of interest (brain, pituitary, heart, liver, spleen, kidneys, skeletal muscle, abdominal white adipose tissue, left femur bone) were dissected, weighed and snap frozen in liquid nitrogen until further processing and analysis.

4.12 Protein Extraction and Quantification

Total protein was extracted from 0.05 g samples of mouse liver in 1.0 ml T-Per Tissue Protein Extraction Reagent (25 mM bicine, 150 mM NaCl, pH 7.6) (Pierce, Rockford, IL) by grinding with a manual mortar and pestle for 2 min. The samples were then allowed to stand for 5 min on ice, followed by a 10 min centrifugation at 10,000 x g at 4°C. The supernatant was then collected. Protease Inhibitor Cocktail (P8340) and Phosphatase Inhibitor Cocktail II (P5726) from Sigma-Aldrich, St. Louis, MO were used as per manufacturer’s instructions to protect extracted proteins from protease and phosphatase action, and the supernatant was aliquoted and stored at -80°C prior to further analysis.

Total protein quantification was performed by the Bradford method using a commercially available Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL). A standard curve was created using a serial dilution of bovine serum albumin (BSA).
Using a 96-well microplate, 5 ul of each standard or unknown sample of liver protein and 250 ul of the Coomassie reagent were added to the appropriate microplate well. The plate was covered and shaken at 450 rpm for 30 sec, then incubated for 10 min at room temperature (RT). Absorbance was read at 595 nm. The average 595 nm measurement for the blank replicates was subtracted from all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average blank-corrected 595 nm measurement for each BSA standard vs. its concentration in ug/ml to determine the protein concentration of each unknown sample.

### 4.13 Enzyme Linked Immuno-Sorbent Assays (ELISAs) and Colorimetric Assays

An Enzyme Linked Immuno-Sorbent Assay (ELISA) is an immunological technique using an enzyme as a label to determine the presence of a specific antigen or target protein in a sample. The enzyme linkage or labeling allows determination of whether the target protein is present (qualification) and at what amount compared to a standard curve (quantification). In the final step of the assay a substrate is added that the enzyme converts to cause a detectable color change if the target protein is present. Plates are then read by a spectrophotometer at wavelengths specified by the manufacturer of the ELISA kits or determined during development of the assay. All samples were run in duplicate for all plates. All plates were read using a SpectraMax 340 (Molecular Devices, Sunnyvale, CA).

1. **Glucose (HK) Assay** (Sigma-Aldrich, Saint Louis, MO). This kit is a colorimetric assay for the quantitative enzymatic determination of glucose as it is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by
hexokinase. Glucose-6-phosphate (G6P) is then oxidized to 6-phospho-glucuronate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to nicotinamidine adenine dinucleotide – reduced form (NADH). The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration. A linear standard curve was created by 5 serial dilutions of glucose standard solution (D-glucose, 1.0 mg/ml in 0.1% benzoic acid). Plasma samples were diluted 1:50 to fit within the expected range for mice.

2. IGF-1 (Mouse, Rat) ELISA protocol and reagents (Alpco Diagnostics, Salem, NH). This product is specifically for the quantitative determination of mouse/rat Insulin-like Growth Factor-1 in serum or plasma. Samples used were plasma, diluted at 1:100 as recommended by the manufacturer. This kit was used to confirm that midi mice had lower plasma IGF-1 than controls.

The IGF-1 ELISA is a sandwich assay. It utilizes two monoclonal specific, high affinity antibodies for this protein. The IGF-1 in the sample binds to the immobilized first antibody on the microtiter plate, the biotinylated and the streptavidin-peroxidase conjugated second specific anti-IGF-1-antibody binds in turn to the immobilized IGF-1. In the closing substrate reaction, a color change is specifically catalyzed; the IGF-1 levels of the samples are then measured.

In order to dissociate IGF-1 from the IGFBPs, the samples were diluted in an acidic buffer. The diluted samples were then pipetted into wells containing the Antibody Conjugate AK thus neutralizing the pH value. After neutralization of
the samples, the excess IGF-II occupies the IGF-binding sites of the binding proteins, thus allowing the measurement of resulting free IGF-1. With this method, the IGFBPs are not removed, but their function and therefore their interference in the assay is neutralized. Due to extremely low cross-reactivity of the IGF-1 antibody with IGF-II, the excess of IGF-II does not disturb the interaction with IGF-1.

3. **Rat/Mouse Insulin Kit** (Millipore, Billerica, MA). This sandwich ELISA assay is based on the capture of insulin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of a monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin. Unbound materials were then washed away from the samples, horseradish peroxidase was bound to the immobilized biotimylated antiboides, free enzyme conjugates were washed away and immobilized antibody-enzyme conjugates were quantified by monitoring horseradish perosidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. The increase in absorbency is directly proportional to the amount of captured insulin in the unknown sample. Therefore, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of rat insulin.

4. **Mouse C-Reactive Protein Quantitation by ELISA** (Helica, Fullerton, CA). C-reactive protein (CRP) is an acute-phase protein produced by the liver in
conditions of inflammation, bacterial infection, or tissue trauma. Quantification of mouse plasma was performed on plasma samples diluted 1:20 as was suggested by the manufacturer and allowed to react with pneumococcal C-polysaccharide coated on microtiter plates. After appropriate incubation, the wells were washed to removed unreacted plasma proteins, and an enzyme-labeled (horseradish peroxidase (HRP)) rabbit anti-CRP-IgG (conjugate) was added to react with and tag the antigen-antibody complexes. Following another incubation period, the wells were washed again to remove unreacted conjugate. A urea peroxide substrate with TMB as chromogen was added to start color development. Blue color development indicated a positive reaction, while negative reactions appeared colorless or with a trace of blue. The reaction was interrupted with a stop solution that turned the blue positive reactions yellow. Absorbance was read at 450 nm. Semi-quantification was accomplished by the use of a standard curve generated by measuring two-fold dilutions of the standard provided (CRP).

**Tissue ELISAs**

1. **Mouse Common Cytokines (Multi-Analyte ELISA Array Kit)** by SABiosciences (Frederick, MD). The Mouse Common Cytokines Multi-Analyte ELISAArray Kit analyzes a panel of 12 important cytokines using a conventional ELISA protocol all at once under uniform conditions. The cytokines represented by this array are IL1A, IL1B, IL2, IL4, IL5, IL6, IL10, IL12, IL13, IL17A, G-CSF, and GM-CSF. IL1A, IL1B, IL2, IL6, IL12, IL17A, and G-CSF, GM-CSF are all pro-inflammatory cytokines. IL4 and IL10 can act as either pro- or anti-inflammatory
cytokines depending on their concentration, while IL13 is exclusively and anti-inflammatory cytokine. IL1 and IL6 are also reported to be endogenous pyrogens. Using the same ELISA protocol and development or incubation time, you can profile the levels of a focused panel of 12 common cytokines with this array.

2. Heat Shock Protein 70 and Heat Shock Protein 90 Assay developed in house.

Liver protein was extracted and quantitated as described above. Each well was loaded with a protein concentration of 10 μg/ml of sample. All chemical reagents were purchased from Sigma-Aldrich, while high binding microtiter ELISA plates, primary and secondary antibodies, and basic protocol were obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Monoclonal HSP70 (made in goat) and polyclonal HSP90α/β (made in goat) assays were developed in the same manner. Briefly, microtiter plates were coated with target protein at 1 μg/ml diluted in 50 mM carbonate buffer at pH 9.0, covered with parafilm and incubated overnight at 4°C. Antigen solution was removed and 200 μl per well SuperBlock Blocking Buffer (Pierce) was added for 15 min. Blocking buffer was removed. Plates were washed once with phosphate buffered saline (PBS), pH 7.4 and tapped smartly on paper towels to remove excess fluid. Samples were diluted into a second microtiter plate, and a standard curve created using BSA. Fifty (50) μl each of samples and standards were transferred onto the prepared microtiter plate using a multichannel pipette to allow more equal incubation time (1 hr). Plates were then washed for three times with PBS containing 0.05% Tween-20, removing excess liquid as before. Then 50 μl/well at a dilution of 1:500 of alkaline phosphatase conjugated secondary antibody (donkey anti-goat IgG AP)
was added and incubated for 1 hr at room temperature. Liquid was then removed from the wells, the plate was washed three times in PBS with 0.05% Tween - 20 and slapped to dry before being washed once in diethanolamine buffer (10 mM diethanolamine, 0.5 mM NaCl, pH 9.5). PNPP substrate was diluted in diethanolamine buffer (1 mg/ml), and 50 ul was added to each well. Color was allowed to develop for approximately 15 min. Reaction was stopped by adding 50 ul of 0.1 M EDTA, pH 7.5. Plates were then read on a microtiter plate read at 405/490 nm.

4.13 Data Management

Microsoft Excel 2007 macro enabled workbooks were used to organize data. The following macro was used to pull data from workbooks in which core body temperature, physical activity, and metabolic information had been separately recorded. Refer to Appendix A for the macro code.

4.14 Statistical Analysis

SAS v.9.1.3 was used to perform all statistical analysis. Survival analysis was used to assess longevity. Descriptive statistics were used to give an overview of data obtained, while t-tests and multi-factorial analysis of variance was performed to analyze the remaining data as appropriate.
Chapter 5 RESULTS

Figure 1. Plasma IGF-1 in control compared to midi mice. Plasma IGF-1 was measured at 180 d of age, and was decreased in midi mice compared to controls, (p < 0.05, n = 20 per group). IGF-1 ELISA kit was used to confirm that midi mice were IGF-1 hypomorphs as expected.
Figure 2. Survival characteristics of control and midi mice. Results indicated sex differences in survival curves as shown as determined by the LIFETEST procedure. Midi mice attained a longer median lifespan, previously defined as the point in time at which 50% of the population had died, than controls (p <0.01). Female midi mice (n = 26) achieved a median lifespan of 768 d which was longer (p < 0.01) than that attained by control female mice (725 d, n = 22). Male control mice attained a median lifespan of 721 d, n = 30, while male midi mice achieved a longer median lifespan of 778 d (p < 0.01). Male midi mice showed an increased maximum lifespan, defined previously as tenth percentile survivors, (897 d) compared to male controls (821 d, p < 0.05), although there was no difference between female midis (813 d) and female controls (822 d), p = 0.11.
Figure 3. **Body weight differences in young and aged mice.** n=40 per age group evenly distributed by sex and genotype. Male and female controls increased in weight (p < 0.05), while male and female midi mice maintained body weight during aging as determined by General Lineralized Model (GLM) procedure (PROC GLM).
Figure 4. Energy expenditure of young and old mice by sex and genotype. n=40 per age group evenly distributed by sex and genotype. From 100 d to 540 d, there was a decrease in total daily energy expenditure (TDEE) (p < 0.03) between midi and control mice, with female mice showing a larger decrease than males (p < 0.05) as determined by PROC GLM.
Figure 5. Physical activity differences in young and old mice by sex and genotype. 

n=40 per age group evenly distributed by sex and genotype. * = p < 0.05, As shown in panel A, there was a trend toward increased physical activity in adulthood until very late life. If physical activity is examined at specific points in early and middle adulthood, such as in panel B, it was seen that control mice did not vary in physical activity by sex or age. However midi mice showed a significant increase in physical activity with age compared to controls. Analyses performed using PROC GLM.
Distance Traveled (meters/hr)

- F Control
- F Midi
- M Control
- M Midi

100 495

160 140 120 100 80 60 40 20 0

B

* denotes significant difference.
Figure 6. Core body temperature differences in young and old mice by sex and genotype. n=40 per age group evenly distributed by sex and genotype. * = p < 0.05, As shown in panel A, all groups core body temperature increased with age except male midi mice as determined by PROC GLM. Female control mice had the greatest increase in body temperature over the lifespan. As shown in panel B, all groups except male midi mice showed an increase in core body temperature from young adulthood to aged adulthood. No core body temperature measurements were obtained at 840 d of age as there were no working implanted e-mitters at this point in the experiment.
Core Body Temperature (°C)

- F Control
- M Control
- F Midi
- M Midi

* indicates significant difference.
Figure 7. Diurnal cycles of energy expenditure, physical activity, and core body temperature in control and midi mice. As shown in panels A, B and C respectively, energy expenditure, physical activity and core body temperature of both control and midi mice followed similar patterns. The 495 d old mice were used as an example; all age groups showed similar patterns.
Figure 8. Percent lean mass in the region of interest (from the cephalic ridge of the scalp blades to the second vertebrae caudal to pelvis) during aging in male and female control and midi mice, n=40 per age group evenly distributed by sex and genotype. There was no change in % lean mass in the region of interest between 100 d and 495 d as determined by PROC GLM.
Figure 9. Lean mass in the region of interest (from the cephalic ridge of the scalpel blades to the second vertebrae caudal to the pelvis) expressed as grams lean mass per grams body weight during aging in male and female control and midi mice, n=40 per age group evenly distributed by sex and genotype. Measurements at age 840 d were obtained via MRI, while previous age measurements were obtained via DEXA.
Figure 10. Percent fat in the region of interest (from the cephalic ridge of the scalpel blades to the second vertebrae caudal to the pelvis) during aging in male and female control and midi mice, n=40 per age group evenly distributed by sex and genotype. Measurements at age 840 d were obtained via MRI, while previous age measurements were obtained via DEXA.
Figure 11. Bone mineral density alterations in aging mice as measured by DEXA, n=40 per age group evenly distributed by sex and genotype. * = p < 0.05, at 100 d of age, BMD was lower (p<0.0001) in midi mice compared to controls, with no effect between sexes of either genotype (p = 0.321). At 495±5 d, BMD was lower in midi mice (p<0.0001) compared to controls. Sex also influenced BMD (p=0.010), with males of both genotypes exhibiting lower BMD in older animals as determined by PROC GLM.
Figure 12. Bone mineral density alterations in aging mice as measured by DEXA, n=40 per age group evenly distributed by sex and genotype. As shown in panel A, BMD was lower (p<0.0001) in the oldest midi mice compared to controls. Sex also influenced BMD (p<0.01), with the oldest control and midi males exhibiting lower BMD than females as determined by PROC GLM. BMD data not available for 840 d of age due to risk of death from anesthesia. As shown in panel B, BMD was positively correlated (R = 0.64, p < 0.01) with body weight throughout life.
Figure 13. Plasma glucose by sex and genotype during aging. Plasma glucose was measured at 100, 180, and 720 d of age, n=40 per age group evenly distributed by sex and genotype. Plasma glucose decreased with age in midi mice compared to controls (p < 0.05), with females having higher plasma glucose than males of the same genotype in late life (p<0.01).
Figure 14. Plasma insulin by sex and genotype during aging. Plasma insulin was measured at 100, 180, and 720 d of age, n=40 per age group evenly distributed by sex and genotype. Plasma insulin was highest in male mice compared to all other groups (p=0.001) at 100 d and 180 d of age, although this effect was not seen at 720 d of age.
Figure 15. Total protein concentration in the livers of heat-shocked mice. There was no difference in total protein content by sex or genotype (p>0.05, n = 36 evenly distributed by genotype and sex).
Figure 16. C-reactive protein (CRP) from liver extraction in control and midi mice

pre-hyperthermia and post-hyperthermia. All groups, n = 36 evenly distributed by genotype and sex, showed significantly more CRP post-hyperthermia (p<0.001) compared to pre-hyperthermia. Control mice expressed more CRP post-hyperthermia than midi mice (p<0.01) as determined by PROC GLM.
Figure 17. Heat shock protein 70 (HSP70) levels in liver from control and midi mice pre-hyperthermia and post-hyperthermia. While pre-hyperthermia HSP70 levels were equal and post-hyperthermia levels increased in all groups, n = 36 evenly distributed by genotype and sex, *(p<0.01), male midi mice showed a comparatively lower response than other groups **(p<0.05).
Figure 18. Heat shock protein 90 (HSP90) levels in liver from control and midi mice pre-hyperthermia and post-hyperthermia. While post-hyperthermia levels, n = 36 evenly distributed by genotype and sex, were higher in all groups compared to pre-hyperthermia levels (p<0.001), no differences were noted by genotype.
Figure 19. Oxidative damage measured as nmols carbonyls per mg liver protein in post-hyperthermia treatment mice. Midi mice had more oxidative damage (p<0.02, n = 9 per group) as measured by Millipore OxyELISA kit than control mice, with no effect of sex (p= 0.43) as determined by PROC GLM.
Figure 20. Mouse common cytokines array indicated no differences in liver cytokine concentration between midi and control mice following whole body hyperthermia.

No differences were found when SABiosciences Multi-Analyte ELISAArray Kits were used to analyze a panel of 12 mouse common cytokines (IL1A (p = 0.19), IL1B (p = 0.76), IL2 (p = 0.52), IL4 (p = 0.15), IL5 (p = 0.82), IL6 (p = 0.73), IL10 (p = 0.78), IL12A (p = 0.40), IL13 (p = 0.48), IL17A (p = 0.59), G-CSF (0.08), GM-CSF (p = 0.41)), n = 6 per group, 3 males and 3 females per genotype.
Figure 21. Core body temperature of mice during whole body hyperthermia experiment. All male midis were removed from the hyperthermia experiment by 22 min; all female controls were removed by 23 min; all female midis were removed by 24 min; all male controls by 26 min as animals had reached criteria for removal as described in the methods section, n = 36 evenly distributed by genotype and sex.
Figure 22. Energy expenditure during whole body hyperthermia in midi and control mice. Male midi mice had the lowest energy expenditure (p<0.01) of all groups, as was seen when the animals were not being stressed.
Chapter 6 DISCUSSION

Murine studies have long contributed to the investigation of humans as aging for both is often defined by signs of changes in phenotype, such as increased risk of frailty, disability, morbidity, and ultimately, mortality. This study was undertaken in part to determine survival characteristics of male and female mice having variable levels of IGF-1 under standard laboratory conditions, as previously described. Figure 1 illustrates that midi mice had lower plasma IGF-1 levels than controls as expected, allowing us to proceed with the remaining experiments. It must be noted that the midi mouse model is an IGF-1 hypomorph with lower IGF-1 in all tissues including blood. Plasma IGF-1 was measured as a matter of convenience. Midi mice were expected to have increased lifespan, in accordance with preliminary evidence shown in the laboratories of our colleagues. As shown in Figure 2, in our test population of 106 mice, midi mice had an increased median lifespan, previously defined as the point in time at which 50% of the population had died, compared to controls. This finding is in accordance with our central hypothesis and the preliminary findings of our colleagues. A recent study by The Jackson Laboratory also supports this finding, having shown a negative correlation of IGF-1 and lifespan in longer lived (over 600 d) mouse strains [46]. Male midi mice also showed a greatly increased maximal lifespan, previously defined as tenth percentile survivors, compared to the other groups. The midi mouse model is strikingly different than the Igfr(+/-) model (inactivated IGF-1 receptor) described by Holzenberger as the females lived 33% longer [58]. Midi mice, which are dwarf counterparts to controls, followed the notion that smaller animals live longer than larger animals [59]. Among mammals, maximum species lifespan potential scales with body mass [60]. Humans, naked mole
rats and bats are among the few animals that live significantly less than predicted allometrically [61]. The body size of organisms on CR is less than that of freely eating animals. This may be important since within a given species (such as horses, dogs, rodents), the longest-lived individuals are frequently those having the smallest body size e.g. [62]. A major determinant of body size is Growth Hormone (GH) and its tissue surrogate, IGF-1 (insulin-like growth factor 1). A striking characteristic of CR in rodents is greatly decreased levels of circulating IGF-1 suggesting the possible critical importance of lowered levels of this hormone in the health-related effects of CR. Evidence for the role of lowered GH and IGF-1 in maintaining health in old age is however lacking and there is apparently no effect of CR on plasma IGF-1 levels in humans [63]. Other characteristic features of the CR state such as decreased levels of oxidative stress, plasma glucose and increased resistance to stress, etc have also been addressed as possible mechanisms of action of CR. However, these have been found to be not essential components of the beneficial effects of CR when tested in isolation of other changes. Examples of such demonstrations are the several reports in mice documenting absence of effects on lifespan and healthspan of genetically-induced levels of increased or decreased oxidative damage, as well as decreased levels of plasma glucose [25, 64]. It seems likely from the many tests of individual characteristic effects of CR that, in fact, it is the altered pattern of metabolism as a whole, rather than the individual changes, which is responsible for the beneficial effects of CR. Given the complex nature of the homeostatic mechanisms responsible for maintaining the health of an entire organism, this conclusion appears eminently reasonable. It appears entirely reasonable that there are multiple pathways concurrently regulating lifespan, and that future research should be
more collaborative to allow further elucidation and evaluation of additional pathways during experiments.

As shown in Figure 3, midi mice maintained body weight during adulthood, showing a decreasing trend at 840 d of age, whereas control animals continued to gain weight through adulthood. Male controls gained weight at a greater rate than other animals, particularly in late life. This effect in late life may be due to the effect of individuals alive and available for random selection. There was also a trend for a decrease in energy requirements, as measured as kcal/g lean mass/24 hrs, of all groups over the lifespan. Energy expenditure was lowest for female midi mice and highest for male controls throughout most of the lifespan, as shown in Figure 4. At 840 d, energy expenditure for male controls decreased and those for female midi mice increased somewhat. This result was unexpected as per specific aim two, as midi mice were expected to have higher energy expenditure than controls. Food consumption was equivalent or higher for midi mice when normalized to grams body weight. Physical activity in adult life was higher for midi mice compared to controls as is shown in Figure 5. These results are consistent with our expectations in specific aim two, as we expected midi mice to be more active than controls. There was a trend toward increasing physical activity in midi mice shown through 540 d of age. However, there was a very steep decline at 840 d of age. Control mice physical activity changed very little over the lifespan. This, combined with the aforementioned control animal results most likely explains the higher body weights in control mice compared to midi mice.

As shown in Figure 6, mean core body temperature over 24 hrs increased in female control, male control, and female midi mice during aging, but were unchanged in
male midi mice. This result cannot be explained by differences in exercise or energy expenditure over 24 hrs. Male midi mice had a lower core body temperature than other groups as shown in panel B of Figure 6, which is of particular note as they also lived longer compared to the other groups. A previous study in which transgenic mice engineered to over-express the uncoupling protein 2 in hypocretin neurons (Hcrt-UCP2) had elevated hypothalamic temperature [38]. The effects of local temperature elevation on the central thermostat resulted in a 0.3 degrees to 0.5 degrees C reduction of the core body temperature [38]. Fed ad libitum, Hcrt-UCP2 transgenic mice had the same caloric intake as their wild-type littermates but had increased energy efficiency and a greater median life span (12% increase in males; 20% increase in females) [38]. Thus, modest, sustained reduction of core body temperature prolonged life span independent of altered diet or CR [38]. To date, this is the only study which has shown that lower body temperature in a mammalian model increases longevity, even though CR reduces CBT and feeding causes transient increases in CBT [65]. Figure 7 shows the normal 24 hour diurnal variation of energy expenditure, physical activity, and core body temperature of all groups. Time 08:00 was randomly assigned as the starting point. It should be noted that all groups followed similar diurnal patterns, as was expected. Additional studies examining the hypothalamic controls of temperature regulation in the midi mouse model would provide worthwhile insight into temperature regulation during aging. It is possible that male midi mice received some additional benefit from having low core body temperature and low IGF-1. Female midi mice may not have had the same benefits due to the estrous cycle [66, 67].
Another goal of this study, as was outlined within specific aim three, was to measure body composition and bone mineral density (BMD) over the lifespan in male and female mice having variable levels of tissue IGF-1. It was predicted that midi mice would maintain a body composition profile more consistent with that of a young animal, maintaining more lean mass and less adipose as a percentage of body weight as compared to control animals. It was expected that midi mice may have a lower bone mineral density due to their lower body weight and lower IGF-1 levels compared to controls. Magnetic Resonance Imaging (MRI) and Dual Energy X-Ray Absorptiometry (DEXA) scanning were used to determine whole body fat and lean masses, with the latter being most useful in determining lean and fat mass for specific region of interest and bone mineral density.

As shown in Figures 8 and 9, at 100 d of age, no difference among groups was seen in terms of g lean mass/g body weight in the region of interest. Through middle life, lean mass fluctuated as a reflection of body weight. In late life male control mice had less g lean mass/g body weight in the region of interest than other groups, as would be expected due to considerable fatness at this stage of life. There was no difference among groups in percent lean mass in the region of interest, defined as the area from the cephalic ridge of the scalpel blades to the second vertebrae caudal to the pelvis, but midi mice did maintain. Figure 10 shows the steady increase in percent fat of male control mice while female control mice increased until very late life, then decreased sharply at 840 d. Midi mice of both sexes had relatively stable percent body fat until after 540 d at which time percent fat declined. As was expected, midi mice had lower bone mineral density compared to controls throughout the study. This finding was as expected in specific aim three. At 100 d of age, sex did not influence BMD. However, at 495 d both control and
midi males exhibited lower BMD than females. It is possible that sex hormones such as estrogens were playing a contributing role, even though the mice were well past their sexual prime. Estrogens play a key role in regulation of bone mass and strength by controlling activity of bone-forming osteoblasts and bone-resorbing osteoclasts [68]. Androgens also affect bone mass and integrity [69], however no measures of sex steroids were taken during this study so we are unable to determine if these factors greatly impacted bone mineral density. Future studies should take this into consideration. Midi mice exhibited lower BMD than controls at 100 d of age, with male midi mice maintaining a BMD lower than other groups until 540 d, at which point it decreased even more in comparison. Bone mineral density was not measured at 840 d due to the potential risk of death due to anesthesia during DEXA scanning.

As shown in Figure 13, plasma glucose was measured at 100, 180 and 180 d of age as per specific aim three. Plasma glucose decreased with age in midi mice compared to controls with females having higher plasma glucose than males of the same genotype in late life. Figure 14 shows plasma insulin levels. Male midi mice had the highest insulin levels in addition to the highest plasma glucose levels at both 100 d and 180 d. While this might be immediately flagged as an unhealthy animal model, it should again be noted that these animals attained the longest maximal lifespan, were among the most physically active during most of the lifespan, had the lowest core body temperature throughout the lifespan, and had an intermediate level of energy expenditure of the four groups. Clearly the hypothesis that elevated plasma insulin is a necessary component of extended longevity is not supported by our data and deserves further detailed study.
Resistance to stress promotes longevity, and in specific aim four we measured response to acute thermal stress in male and female mice having different tissue levels of IGF-1. Acute stress response was induced via whole body hyperthermia (WBH) as was previously described. Total protein was extracted from the livers of mice from the WBH experiment and an equal number who had not undergone thermal stress. C-reactive protein (CRP) was compared in livers of control and midi mice, as shown in Figure 16. There was no difference among groups who had not undergone thermal stress. All groups showed a within group increase in CRP post-hyperthermia (p<0.001). Control mice expressed more CRP compared to midi mice (p<0.01), with male midi mice having the lowest response of all groups. High circulating levels of CRP have been associated with human mortality [70, 71]. In the recently published Rancho Bernardo study, 610 men and 743 postmenopausal women were followed for mortality for up to 23 years. Higher levels of inflammatory markers including IL-6 and CRP were associated with decreased survival time and lifespan [72]. Our findings are consistent with human literature, as the male midi mice lived longer than female midi mice and male and female controls. These results support the hypothesis that decreased response to stress is associated with extended longevity. They also support the use of response to stress as a biomarker of aging.

Heat shock proteins (HSPs) serve as molecular chaperones and endogenous cytoprotective factors [73]. Heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90) are ubiquitously expressed and have been well studied in relation to aging and resistance to stress and were therefore chosen for this study as we examined the midi mouse model as a novel approach to examining longevity and thermal stress. When not
interacting with a substrate peptide, Hsp70 is primarily found in an ATP bound state. HSP70 is characterized by very weak ATPase activity, such that spontaneous hydrolysis will not occur for several minutes. The substrate binding domain of HSP70 recognizes sequences of hydrophobic amino acid residues as they emerge from ribosomes, spontaneously interacting in a reversible fashion allowing HSP70 to relatively freely bind and release peptides. However, the presence of a peptide in the binding domain stimulates the ATPase activity of HSP70, increasing its normally-slow rate of ATP hydrolysis. When ATP is hydrolyzed to ADP the binding pocket of HSP70 closes, tightly binding the now-trapped peptide chain. These co-chaperones dramatically increase the ATPase activity of HSP70 in the presence of interacting peptides. By binding tightly to partially-synthesized peptide sequences (incomplete proteins), HSP70 prevents them from aggregating and being rendered nonfunctional. Once the entire protein is synthesized, a nucleotide exchange factor (BAG-1 and HSPBP1 are among those which have been identified) stimulates the release of ADP and binding of fresh ATP, opening the binding pocket. The protein is then free to fold on its own, or to be transferred to other chaperones for further processing. HOP (the HSP70/HSP90 Organizing Protein) can bind to both HSP70 and HSP90 at the same time, and mediates the transfer of peptides from HSP70 to HSP90 [40]. HSP90 also stabilizes the 26S proteasome and several kinases, and has been shown to be an important therapeutic target in cancer treatment [74]. As shown in Figure 18, there were no differences among groups in pre-hyperthermia HSP90 levels, and all groups showed an increase following whole body hyperthermia (p<0.001). As shown in Figure 17, there were no differences among groups in pre-hyperthermia HSP70 levels, although there was an increase among all groups post-
hyperthermia compared to pre-hyperthermia. Male midi mice showed a comparatively lower response than all other groups (p<0.05). Male midi mice lived longer and had lower biomarkers of stress, HSP70 and CRP, following whole body hyperthermia, indicating increased resistance to stress compared to controls and female midis.

In 1956 Denham Harman proposed the free radical theory of aging, in which endogenously generated free radical reactions were hypothesized as the cause of aging [75]. Free radicals are produced in the course of usual cellular metabolism and have deleterious side effects on cell constituents, leading Harman to suggest hydroxyl and superoxide radicals would impair functional efficiency of the cell by damage to nucleoproteins and nucleic acids, in turn reducing reproductive ability of the cell. Harman also hypothesized that oxygen catalysts such as iron, cobalt, manganese interacting with superoxide in the intracellular spaces would affect the integrity of connective tissues. He later predicted that the rate of oxidation at any given time depends on the age, or in other words, the overall extent of oxidation of the body [76]. This study compared serum mercaptan levels of 35 normal individuals aged 20 to 60 years to those of 9 individuals over 60 years of age, and indicated that free radical oxidation reactions may occur in the serum of elderly patients at a greater rate than in young patients. While Harman declared the decrease seen in mercaptan levels with age is not necessarily due to an increased rate of oxidation as postulated by the free radical aging theory, he concluded that decreased mercaptan levels with age indicated, with all other factors remaining constant, that free radical oxidation reactions may occur at a greater rate in aged individuals versus young individuals [76].
More recently, this theory has focused on oxygen radical production in mitochondria, and is often referred to as the Mitochondrial Free Radical Theory of Aging (MFRTA) [77-79]. MFRTA proposes that mitochondrial free radicals, produced as by-products of usual metabolism, cause oxidative damage. According to MFRTA, the accumulation of this oxidative damage is the main driving force in the aging process [80]. Potential sources of intracellular reactive oxygen species (ROS) include the mitochondrial electron transport chain, xanthine oxidase, and polymophonuclear leukocytes (PMNs or neutrophils) [81]. Mitochondria, responsible for more than 90% of oxygen use in cells, are a major source of intracellular ROS [82]. Approximately 1-2% of oxygen consumed by mitochondria is converted to superoxide and hydrogen peroxide, which are then metabolized by the Mn-containing superoxide dismutase (SOD) and the Se-containing glutathione peroxidase (GPX), respectively [82]. It has been suggested that the only way to test the mitochondrial free radical theory of aging is by specifically decreasing mitochondrial free radical production (mtROS) without altering other physiological parameters such as insulin signaling [80]. It would be expected that animals producing fewer mtROS would live longer than experimental controls [80]. However this expectation has rarely been realized when tested [64]. Xanthine oxidase is a flavoprotein enzyme which catalyzes the oxidative hydroxylation of purine substrates, and is a well-established target of drugs against gout and hyperuricemia. In some pathologies, xanthine oxidase levels increase, increasing free radical production and causing damage to surrounding tissues [83]. Neutrophils also play a role in human immunity, as they are the most abundant innate immune cell and kill most invading bacteria through combined activities of ROS and antimicrobial granule constituents [84].
Reactive oxygen species such hydroxyl (•OH), superoxide (O$_2^{•-}$), hydrogen peroxide (H$_2$O$_2$), peroxyl (ROO•), alkoxyl (RO•), phenoxy (ArO•) and semiquinone (HO-Ar-O•) radicals are the most abundant oxygen centered free radicals in biological systems [85]. The hydroxyl radical, the most reactive oxygen containing species, generally interacts with biologicals primarily by electron transfer, addition and abstraction mechanisms. The oxidation of metals, such as copper and iron, and biological complexes such as proteins containing these metals, may be the most damaging action of •OH. Oxidation of organic sulfides, in which •OH adds to the lone p-electron pair of sulfur, has also been studied in depth and is one route by which the essential amino acid methionine undergoes oxidative decarboxylation. Pyrimidine bases, vital constituents of DNA, are also a target of •OH. Peroxyl radicals result from oxygen addition to carbon centered free radicals and are thought to be the most abundant oxygen centered free radical in biological systems. Lipid peroxidation, specifically that of polyunsaturated fatty acids, is often caused by ROO•. Organic sulfates, again including the essential amino acid methionine, are targeted by ROO•. Metabolic degradation of halogenated organic material via ROO• intermediates yields aldehydes and acids, products which may have toxic side effects such as inducing pH changes. Alkoxyl radicals have a half-life of about a microsecond and are oxidizing species. RO• can demonstrate 1,2-hydrogen shift and a β-scission process, changing the redox properties of the radical and shortening the carbon chain by one carbon unit, respectively. When the aliphatic (R) is replaced by an aromatic substituent (Ar), a phenoxy radical is formed. Oxidation of ascorbate (Vit C) is readily performed when the ArO• α-tocopherol (Vit E) interact, creating a chromanoxyl radical. Semiquinone radical, under which the chromanoxyl radical may be classified, are
special phenoxy radicals and may contribute to superoxide formation in biological systems. Under physiological conditions, \( \text{O}_2^* \) is a reductant and transfers an electron to a recipient such as cytochrome-c, although it is a fairly stable species by itself and may be transported or interact with long pathways [86].

While production of ROS may lead to RNA, DNA, protein and lipid damage or cellular apoptosis, ROS have also been suggested to play a positive role, such as in cell signaling and in regulation of gene expression [87-91]. \( \text{H}_2\text{O}_2 \) has been implicated as a second messenger that mediates signal transduction by chemoselective oxidation of cysteine residues in signaling proteins [92]. \( \text{H}_2\text{O}_2 \) can be quickly generated in cells, selectively perceived by downstream proteins, and undergo degradation by cellular antioxidant defense systems. Collectively, these properties make \( \text{H}_2\text{O}_2 \) an ideal mediator of signal transduction processes. The thiol side chain of cysteine residues are susceptible to oxidation by \( \text{H}_2\text{O}_2 \), with the initial reaction of the substrates producing a sulfenic acid (Cys-SOH) [92]. Cys-SOH has important roles as a catalytic center and as a sensor of oxidative and nitrosative stress in enzymes and transcriptional regulators [93]. Protein sulfenic acids are widespread physiologically relevant posttranslational oxidative modifications that can be detected at basal levels in healthy tissue, and are elevated in response to hydrogen peroxide [94]. The thioredoxin system is currently a target for cancer chemotherapy [95]. ROS are important cellular messengers in signal transduction involved in the activation of a wide range of transcription factors such as redox sensitive nuclear factor (NF)-\( \kappa \)B and AP1 (activator protein 1) [96]. NF-\( \kappa \)B activation was recently found to highly correlate with the production of interferon lambda, important in optimal antiviral protection [97]. Inhibition of NF-\( \kappa \)B transcription factor by A20 in the
tumor necrosis factor-receptor and toll-like receptor pathways has recently been shown to negatively regulate inflammation, although the exact mechanism is still unclear [98]. Recently, short term (10 d) 40% caloric restriction (CR) in aged rats inhibited binding activity of NF-κB and AP1 as well as the thioredoxin/Ref-1 pathway. Therefore, expression of NF-κB and AP1 responsive genes COX-2, iNOS, VCAM-1, and ICAM-1 was also blocked [99]. It is obvious from these examples that essential intracellular signaling requires ROS production, recognition by oxidation sensitive factors such as NF-κB, followed by gene regulation. Oxidative free radicals therefore function as both necessary agents in the maintenance of health but are also viewed as primary agents of damage.

Vitamins A, C and E, coenzyme Q10, and resveratrol are well known as having antioxidant capacities, protecting cells from ROS damage. Dietary fruits and vegetables provide a variety of chemicals that act as physiological antioxidants and are highly recommended as part of a healthy diet [100]. A large French study, The Supplementation en Vitamines et Mineraux Antioydants (SU.VI. MAX) was a randomized, double-blind, placebo-controlled primary prevention trial in which 13,017 French adults (7876 women aged 35-60 yrs and 5141 men aged 45-60 yrs) were included. The experimental group took a single daily capsule of a combination of 120 mg of ascorbic acid, 30 mg of Vit E, 6 mg of beta carotene, 100 μg of selenium, and 20 mg of zinc, while the remaining subjects took a daily placebo capsule. Median follow-up time was 7.5 years. No significant differences were detected between the groups in total cancer incidence, ischemic cardiovascular disease incidence, or all-cause mortality [101]. However, sex-stratified analysis showed a protective effect of antioxidant supplementation in men but
not in women [101]. The authors suggested that the low dose supplementation in this experiment was effective only in men due to their lower baseline antioxidant status of some nutrients, particularly of beta carotene. Postintervention follow-up assessment of total cancer incidence, ischemic cardiovascular disease incidence, and total mortality was carried out for 5 years after the SUVI-MAX study. No late effect of antioxidant supplementation was revealed 5 years after ending the intervention neither on ischemic cardiovascular disease incidence and mortality in both genders nor on cancer incidence in women[102]. Regarding duration of intervention effects in men, the reduced risk of total cancer incidence and total mortality was no longer evident after the 5-year post-intervention follow-up. In conclusion, beneficial effects of antioxidant supplementation in men disappeared during post-intervention follow-up [102]. Deficiencies of vitamins A, C, and E have been implicated in cases of cervical cancer in Korean women [103].

Another large experiment was The General Population Nutrition Intervention Trial in China, in which 29,584 adult participants in Linxian, China were given daily vitamin and mineral supplements. “Factor D” as the supplements were named a combination of 50 μg of selenium, 30 mg Vit E, and 15 mg beta-carotene. This randomized primary esophageal and gastric cancer prevention trial was conducted over 6 yrs, with decreased mortality from all causes, cancer overall and gastric cancer [104]. Ten years postintervention, beneficial effects of selenium, Vit E, and beta-carotene on mortality were still evident and were consistently greater in younger participants [104]. Contrasting information from the French and Chinese trials indicate supplement combination and other factors may greatly impact the benefits or risks of antioxidant supplementation.
Coenzyme Q10 plays a fundamental role in mitochondrial bioenergetics and its well-acknowledged antioxidant properties constitute the basis for its clinical applications. Reduced muscle levels of coenzyme Q10 are frequent in patients with mitochondrial myopathy [105], and low levels of plasma coenzyme Q10 are associated with cardiovascular disease, although it is unclear as to whether or not coenzyme Q10 supplementation can effectively lower blood pressure [106]. However, coenzyme Q10 supplementation has been shown to lower hepatic oxidative stress and inflammation associated with diet-induced obesity in mice [107]. Trans-resveratrol or (E)-resveratrol [3,4′,5 trihydroxy-trans-stilbene, t-RESV or (E)-RESV] is a natural component of Vitis vinifera L. (Vitaceae), abundant in the skin of grapes (but not in the flesh) and in the leaf epidermis and present in wines (especially red wines). In vitro, ex vivo and in vivo experiments, t-RESV exhibits a number of biological activities, including anti-inflammatory, antioxidant, platelet antiaggregatory and anticarcinogenic properties, and modulation of lipoprotein metabolism [108]. Some of these activities have been implicated in the cardiovascular protective effects attributed to t-RESV and to red wine. t-RESV has been the subject of intense interest in recent years due to a range of unique anti-aging properties. These include cardiovascular benefits via increased nitric oxide production, down-regulation of vasoactive peptides, lowered levels of oxidized low-density lipoprotein, and cyclooxygenase inhibition; possible benefits on Alzheimer's disease by breakdown of beta-amyloid and direct effects on neural tissues; phytohormonal actions; anticancer properties via modulation of signal transduction, which translates into anti-initiation, antipromotion, and antiprogression effects; antimicrobial effects; and sirtuin activation, which is believed to be involved in the
caloric restriction-longevity effect [109]. It appears as though coenzyme Q10 and t-RESV effects are due at least in part to reduced inflammation, similar to findings of studies involving NF-κB. More studies integrating the effects of multi-antioxidant supplementation at various ages could continue to explore the role of inflammation in aging.

Current findings in transgenic mouse models indicate that ROS damage attenuated by decreased production of free radicals and increased resistance to various forms of oxidative stress did not lead to increased lifespan [110]. Indeed the same research group has pointed out "Our research with 18 different genetic manipulations in the antioxidant defense system show that only the mouse model null for Sod1 had an effect on lifespan that would be predicted from the oxidative stress theory of aging" [64]. As shown in Figure 19, our results clearly align with the findings of these studies, as midi mice had higher levels of carbonyls per mg liver protein and yet lived longer than controls. Such results suggest the oxidative free radical theory may not have general applicability in explaining mechanisms of aging in transgenic models.

Cytokines, a category of signaling molecules used extensively in cellular communication, are small proteins, peptides, or glycoproteins secreted by specific glial and immune cells [111]. No differences were found when SABiosciences Multi-Analyte ELISArray Kits were used to analyze a panel of 12 mouse common cytokines (IL1A (p = 0.19), IL1B (p = 0.76), IL2 (p = 0.52), IL4 (p = 0.15), IL5 (p = 0.82), IL6 (p = 0.73), IL10 (p = 0.78), IL12A (p = 0.40), IL13 (p = 0.48), IL17A (p = 0.59), G-CSF (0.08), GM-CSF (p = 0.41)), n = 6 per group, 3 males and 3 females per genotype. These
experiments were run as a profiling mechanism to assist in identifying additional pathways that may be important in whole body hyperthermia. It is possible that there was inadequate time between whole body hyperthermia and organ harvesting for production of cytokines. As shown in Figure 21 core body temperature increased in all groups with the exception of female controls during whole body hyperthermia. Figure 22 shows energy expenditure during whole body hyperthermia, with moderate fluctuation shown only by male midis.
Health in late life is the net result of a complex interplay of biological, psychosocial and environmental factors occurring over a lifetime. The central hypothesis was that lifelong decreased IGF-1 promotes a decrease rate of aging, in part through an enhanced resistance to stress. Midi mice attained a longer median lifespan than controls, and male midi mice attained a longer maximal lifespan than all other groups. Our findings were in accordance with the central hypothesis and with the preliminary findings of our collaborators. As was examined in specific aim two, midi mice maintained adult weight and fat mass until very late life in comparison to controls. Note the increase in weight in male and female controls from 100 d to 495 d of age, while male and female midi mice maintain approximately the same body weight. At 100 d of age, energy expenditure was approximately twice that of 495 d old mice. Control mice did not vary in physical activity by sex or age, while midi mice showed a significant increase in physical activity with age. Core body temperature increased with age in all groups with the exception of male midi mice. At 100 d of age, BMD was lower in midi mice compared to controls, with no effect between sexes of either genotype. At 495 d, BMD was lower in midi mice compared to controls. Sex also influenced BMD in older animals, with males of both genotypes exhibiting lower BMD.

Total protein was extracted from liver of mice. There was no difference in liver total protein content by sex or genotype. All mice expressed similar non-stress levels of HSP70 and HSP90, both of which are ubiquitously expressed. Following hyperthermia, male midi and male control mice produced the highest levels of liver HSP90, followed by
female midis. Female control mice produced the lowest level of liver HSP90 following the hyperthermia stressor. Following hyperthermia, midi mice expressed slightly higher levels of liver HSP70 than controls. Liver C-reactive protein was minimally expressed in all groups in a non-stressful environment. Following hyperthermia treatment, liver CRP levels were significantly higher in control animals than midis. Liver CRP was higher in female midis than male midis. Interestingly, midi mice had higher liver oxidative damage than controls indicating oxidative damage may not be as useful as previously thought, at least in transgenic models. There was no difference between control and midi mice in any of the twelve cytokines tested in liver. These experiments demonstrate the use of the midi mouse as a model to test theories of aging and factors involved in maintenance of healthspan, in particular those involving inflammation, stress, nutrition, exercise, and innumerable others. The data demonstrate also the validity of the hypothesis that tissue igf-1 levels modulate aging processes.
REFERENCES


97. Iversen, M.B., et al., *Expression of type III IFN in the vaginal mucosa is mediated primarily by DCs and displays stronger dependence on NF-κB than type I IFNs.* J Virol, 2010.


Appendix

'This macro takes in data files from numerous sources and consolidates into one sheet that can then be used to calculate data more efficiently.

Option Explicit

'Declaration of BrowseInfo type for use in the browse for folder function

Private Type BrowseInfo
    hwndOwner As Long
    pidlRoot As Long
    sDisplayName As String
    sTitle As String
    ulFlags As Long
    lpfn As Long
    lParam As Long
    iImage As Long
End Type

'Allows the use of dialogs to browse for a folder
Private Declare Function SHBrowseForFolder Lib "Shell32.dll" (bBrowse As BrowseInfo) As Long

Private Declare Function SHGetPathFromIDList Lib "Shell32.dll" (ByVal lItem As Long, ByVal sDir As String) As Long

Sub ConsolidateDataFiles()

    Dim directoryToScan As String

    Dim SelectedFile As String, MainFileName As String, FileName As String

    Dim MRFile As Variant

    'Pick the base directory that has the data files in it.
    directoryToScan = BrowseForDirectory()

    If directoryToScan <> "" Then

        Dim files() As String

        'Search for files in the MRData directory and create a new file based on the filename that will be the basis of the consolidated data for that file
        If (FindFiles(directoryToScan + "\MRData\", "xls", files) = True) Then

            For Each MRFile In files

                Workbooks.Add

            Next

        End If

    End If

End Sub
MainFileName = FileNameNoExt(CStr(MRFile)) & 
"-f.xls"

ActiveWorkbook.SaveAs FileName:=directoryToScan & "\" & 
MainFileName, FileFormat:=xlExcel8, Password:="", WriteResPassword:="", 
ReadOnlyRecommended:=False, CreateBackup:=False

Sheets.Add After:=Sheets(Sheets.Count)

Sheets.Add After:=Sheets(Sheets.Count)

Sheets("Sheet1").Name = "MetabolicSubjectInfo"

Sheets("Sheet2").Name = "MetabolicData"

Sheets("Sheet3").Name = "PhysicalActivity"

Sheets("Sheet4").Name = "TemperatureData"

Sheets("Sheet5").Name = "FinalData"

Sheets("MetabolicSubjectInfo").Select

'Copy in metabolic data

FileName = MRFile

If FileThere(directoryToScan & "\MRData\" & FileName) Then

    Workbooks.Open FileName:=directoryToScan & "\MRData\" & 
    FileName
Sheets("SubjectData").Select

Cells>Select

Selection.Copy

Windows(MainFileName).Activate

ActiveSheet.Paste

Windows(FileName).Activate

Sheets("MetabolicData").Select

Cells.Select

Selection.Copy

Windows(MainFileName).Activate

Sheets("MetabolicData").Select

ActiveSheet.Paste

Application.CutCopyMode = False

Windows(FileName).Activate

ActiveWindow.Close

Sheets("MetabolicData").Select
'Perform additional calculations for Metabolic data

Columns("J:J").Select

Selection.Insert Shift:=xlToRight,
CopyOrigin:=xlFormatFromLeftOrAbove

Columns("L:L").Select

Selection.Insert Shift:=xlToRight,
CopyOrigin:=xlFormatFromLeftOrAbove

Selection.Insert Shift:=xlToRight,
CopyOrigin:=xlFormatFromLeftOrAbove

Selection.Insert Shift:=xlToRight,
CopyOrigin:=xlFormatFromLeftOrAbove

Range("J1").Value = "VdotO2"

Range("L1").Value = "VdotCO2"

Range("M1").Value = "RQ"

Range("N1").Value = "kcal per RQ"
Range("O1").Value = "kcal per min"

Range("J2").Formula = "=(0.2093-(I2/100))*0.431"

Range("J2").Select

Selection.AutoFill Destination:=Range("J2:J1442"), Type:=xlFillDefault

Range("L2").Formula = "=((K2/100)-0.0003)*0.431"

Range("L2").Select

Selection.AutoFill Destination:=Range("L2:L1442"), Type:=xlFillDefault

Range("M2").Formula = "=L2/J2"

Range("M2").Select

Selection.AutoFill Destination:=Range("M2:M1442"), Type:=xlFillDefault

Range("N2").Formula = "=(M2*1.2317)+3.8153"

Range("N2").Select

Selection.AutoFill Destination:=Range("N2:N1442"), Type:=xlFillDefault
Range("O2").Formula = "=(0.2093*N2)"

Range("O2").Select

Selection.AutoFill Destination:=Range("O2:O1442"),
Type:=xlFillDefault

End If

'Copy in physical activity data

FileName = FileNameNoExt(CStr(MRFile)) & ".csv"

Sheets("PhysicalActivity").Select

If FileThere(directoryToScan & "\PAData\" & FileName) Then

    Workbooks.Open FileName:=directoryToScan & "\PAData\" & FileName

    Cells.Select

    Selection.Copy

    Windows(MainFileName).Activate

    ActiveSheet.Paste

    Application.CutCopyMode = False
Windows(FileName).Activate

ActiveWindow.Close

End If

'Copy in temperature data

'Temperature data is an ascii data file that can be imported using the import from text file functionality of excel

FileName = FileNameNoExt(CStr(MRFile)) & ".asc"

Sheets("TemperatureData").Select

If FileThere(directoryToScan & "\TData\" & FileName) Then

    Workbooks.OpenText FileName:=directoryToScan & "\TData\" & FileName, Origin:=437, StartRow:=15, DataType:=xlDelimited, TextQualifier:=xlDoubleQuote, ConsecutiveDelimiter:=False, Tab:=False, Semicolon:=False, Comma:=True, Space:=False, Other:=False, FieldInfo:=Array(Array(1, 1), _
Array(2, 1), Array(3, 1), TrailingMinusNumbers:=True

Cells.Select

Selection.Copy

Windows(MainFileName).Activate

ActiveSheet.Paste

Application.CutCopyMode = False

Windows(FileName).Activate

ActiveWindow.Close

End If

Sheets("MetabolicSubjectInfo").Select

Dim MouseID As String, MouseSex As String, StudyDate As String,
MouseGenotype As String

Dim StudyStartTime As Date

Dim MouseHeight As Double, MouseWeight As Double

Dim MouseAge As Integer

'Setup Some variables that will store values we will use later on
MouseID = Range("B3").Value

MouseSex = Range("B5").Value

MouseHeight = Range("B6").Value * 2.54

MouseWeight = Range("B7").Value * 453.59237

MouseAge = Range("B4").Value

MouseGenotype = ""

'Determine what type of mouse whether midi or control

If InStr(LCase$(Range("B2").Value), "midi") > 0 Then

    MouseGenotype = "midi"

End If

If InStr(LCase$(Range("B2").Value), "control") > 0 Then

    MouseGenotype = "control"

End If

StudyDate = Range("B9").Value

StudyStartTime = Range("B10").Value
'Setup final data sheet which will have all the data for that particular mouse

Sheets("FinalData").Select

Range("A1").Value = "Subject Info"
Range("A2").Value = "ID"
Range("B2").Value = "Sex"
Range("C2").Value = "Genotype"
Range("D2").Value = "Age"
Range("E2").Value = "Weight"
Range("F2").Value = "Length"
Range("G2").Value = "Date"
Range("H2").Value = "Hour"
Range("I1").Value = "MetabolicData"
Range("I2").Value = "RER"
Range("J2").Value = "MixO2%"
Range("K2").Value = "VdotO2"
Range("L2").Value = "'MixCO2%"

Range("M2").Value = "'VdotCO2"

Range("N2").Value = "'RQ"

Range("O2").Value = "'kcal per RQ"

Range("P2").Value = "'kcal per min"

Range("Q2").Value = "'MR"

Range("R1").Value = "'PhysicalActivity"

Range("R2").Value = "'DTcm"

Range("S2").Value = "'RTSec"

Range("T2").Value = "'STSec"

Range("U2").Value = "'ATSec"

Range("V2").Value = "'BSM"

Range("W2").Value = "'HC"

Range("X2").Value = "'AC"

Range("Y2").Value = "'V1C"
Range("Z1").Value = "TemperatureData"

Range("Z2").Value = "Temp"

Dim Count As Integer, hour As Integer

Count = 0

'Figure out the starting hour depending on the first measurement after 7am

'eg hour 0=7am, hour 1=8am etc

hour = (DatePart("h", StudyStartTime) - 7) + 1

'Loop for a 24 hour period, anything over 24 hours is ignored

Do While Count < 24

    Dim mStartPoint, mEndPoint, pStartPoint, pEndPoint, tStartPoint, tEndPoint As Integer

    Dim rowCount As Integer

    rowCount = Count + 3

    If Count = 0 Then

        'Determine startpoints of the data to consolidate
mStartPoint = 2

mEndPoint = 61

pStartPoint = 16

pEndPoint = 134

tStartPoint = 2

tEndPoint = 61

Else

'Determine startpoints of the data to consolidate

mStartPoint = 2 + (60 * Count)

mEndPoint = 61 + (60 * Count)

pStartPoint = 16 + (120 * Count)

pEndPoint = 134 + (120 * Count)

Dim i As Integer

For i = pStartPoint To pEndPoint

    If Trim(Range("PhysicalActivity!C" & i).Value) = "Totals:"

Then
pEndPoint = i - 2

Exit For

End If

Next i

tStartPoint = 2 + (60 * Count)

tEndPoint = 61 + (60 * Count)

End If

Range("A" & rowCount).Value = MouseID

Range("B" & rowCount).Value = MouseSex

Range("C" & rowCount).Value = MouseGenotype

Range("D" & rowCount).Value = MouseAge

Range("E" & rowCount).Value = MouseWeight

Range("F" & rowCount).Value = MouseHeight

Range("G" & rowCount).Value = StudyDate

Range("H" & rowCount).Value = hour
'Range("G" & rowCount).Value = ":AVERAGE(MetabolicData!S" & mStartPoint & ":S" & mEndPoint & ")" 'RER

'Range("H" & rowCount).Value = "=AVERAGE(MetabolicData!I" & mStartPoint & ":I" & mEndPoint & ")" 'MixO2

'Range("I" & rowCount).Value = "=AVERAGE(MetabolicData!J" & mStartPoint & ":J" & mEndPoint & ")" 'VdotO2

'Range("J" & rowCount).Value = "=AVERAGE(MetabolicData!K" & mStartPoint & ":K" & mEndPoint & ")" 'MixCO2

'Range("K" & rowCount).Value = "=AVERAGE(MetabolicData!L" & mStartPoint & ":L" & mEndPoint & ")" 'VdotCO2

'Range("L" & rowCount).Value = "=AVERAGE(MetabolicData!M" & mStartPoint & ":M" & mEndPoint & ")" 'RQ

'Range("M" & rowCount).Value = "=AVERAGE(MetabolicData!N" & mStartPoint & ":N" & mEndPoint & ")" 'kcal per RQ

'Range("N" & rowCount).Value = "=AVERAGE(MetabolicData!O" & mStartPoint & ":O" & mEndPoint & ")" 'kcal per min

'Perform calculations on the data
Range("I" & rowCount).Value = "=SUM(MetabolicData!$S" & mStartPoint & ":$S" & mEndPoint & ")/(SUMPRODUCT((MetabolicData!$S" & mStartPoint & ":$S" & mEndPoint & ">0)*1))" 'RER

Range("J" & rowCount).Value = "=SUM(MetabolicData!$I" & mStartPoint & ":$I" & mEndPoint & ")/(SUMPRODUCT((MetabolicData!$I" & mStartPoint & ":$I" & mEndPoint & ">0)*1))" 'MixO2

Range("K" & rowCount).Value = "=(0.2093-(J" & rowCount & "/100))*0.431" 'VdotO2

Range("L" & rowCount).Value = "=SUM(MetabolicData!$K" & mStartPoint & ":$K" & mEndPoint & ")/(SUMPRODUCT((MetabolicData!$K" & mStartPoint & ":$K" & mEndPoint & ">0)*1))" 'MixCO2

Range("M" & rowCount).Value = "=(L" & rowCount & "/100)-0.0003)*0.431" 'VdotCO2

Range("N" & rowCount).Value = "=M" & rowCount & "/K" & rowCount 'RQ

Range("O" & rowCount).Value = "=(N" & rowCount & "+1.2317)+3.8153" 'kcal per RQ

Range("P" & rowCount).Value = "=(0.2093*O" & rowCount & ")" 'kcal per min
Range("Q" & rowCount).Value = 
"=(P" & rowCount & "/E" & rowCount & ")^0.75" 'MR

'Avergae of the physical activity data

Range("R" & rowCount).Value = 
"=AVERAGE(PhysicalActivity!E" & pStartPoint & ":E" & pEndPoint & ")" 'DTcm

Range("S" & rowCount).Value = 
"=AVERAGE(PhysicalActivity!F" & pStartPoint & ":F" & pEndPoint & ")" 'RTSec

Range("T" & rowCount).Value = 
"=AVERAGE(PhysicalActivity!G" & pStartPoint & ":G" & pEndPoint & ")" 'STSec

Range("U" & rowCount).Value = 
"=AVERAGE(PhysicalActivity!H" & pStartPoint & ":H" & pEndPoint & ")" 'ATSec

Range("V" & rowCount).Value = 
"=AVERAGE(PhysicalActivity!I" & pStartPoint & ":I" & pEndPoint & ")" 'BSM

Range("W" & rowCount).Value = 
"=AVERAGE(PhysicalActivity!J" & pStartPoint & ":J" & pEndPoint & ")" 'HC

Range("X" & rowCount).Value = 
"=AVERAGE(PhysicalActivity!K" & pStartPoint & ":K" & pEndPoint & ")" 'AC

Range("Y" & rowCount).Value = 
"=AVERAGE(PhysicalActivity!L" & pStartPoint & ":L" & pEndPoint & ")" 'V1C
Range("Z" & rowCount).Value =
"=AVERAGE(TemperatureData!C" & tStartPoint & ":C" & tEndPoint & ")"

Count = Count + 1

hour = hour + 1

If hour > 24 Then

    hour = 1

End If

Loop

ActiveWorkbook.Close SaveChanges:=True

Next MRFile

End If

'Now all data files have been saved, now create a new file that will take the final data from the different data files and consolidate into one file that can be then processed.

If (FindFiles(directoryToScan & ", "xls", files) = True) Then

Worbooks.Add
ActiveWorkbook.SaveAs FileName:=directoryToScan & "\SellData.xls",
FileFormat:=xlExcel8, Password:="", WriteResPassword:="",
ReadOnlyRecommended:=False, CreateBackup:=False

Sheets("Sheet1").Name = "CompleteData"

Range("A1").Value = "Subject Info"

Range("A2").Value = "ID"

Range("B2").Value = "Sex"

Range("C2").Value = "Genotype"

Range("D2").Value = "Age"

Range("E2").Value = "Weight"

Range("F2").Value = "Length"

Range("G2").Value = "Date"

Range("H2").Value = "Hour"

Range("I1").Value = "MetabolicData"

Range("I2").Value = "RER"

Range("J2").Value = "MixO2%"
Range("Z1").Value = "TemperatureData"

Range("Z2").Value = "Temp"

Dim lastRow As Integer

lastRow = 3

'Find each mouse datafile and copy its data into the new spreadsheet

For Each MRFile In files

    FileName = MRFile

    Workbooks.Open FileName:=directoryToScan & "\" & FileName

    Sheets("FinalData").Select

    Dim startPoint As Integer, endPoint As Integer, j As Integer

    startPoint = 3

    For j = startPoint To startPoint + 30

        If Range("A" & j).Value = "" Then

            endPoint = j

            Exit For

        End If

    Next j

    'Copy data from MRFile into FinalData sheet

    'Paste data from MRFile into FinalData sheet

End If
Next j

Range("A3:Z" & endPoint - 1).Select

Selection.Copy

Windows("SellData.xls").Activate

Range("A" & lastRow).Select


Application.CutCopyMode = False

Windows(FileName).Activate

ActiveWorkbook.Close

lastRow = lastRow + j - 3

Next MRFile

Columns("G:G").Select

Selection.NumberFormat = "m/d/yyyy"

'Replace and DIV/0 with a . for use in stats software

Cells.Replace What:="#DIV/0!", Replacement:=".", LookAt:=xlPart, _
SearchOrder:=xlByRows, MatchCase:=False, SearchFormat:=False, _ ReplaceFormat:=False

ActiveWorkbook.Save

End If

End If

End Sub

'Get the filename with the extension included

Function FileNameWithExt(strPath As String) As String

    FileNameWithExt = Mid$(strPath, InStrRev(strPath, ")") + 1)

End Function

'Get the filename without the extension included

Function FileNameNoExt(strPath As String) As String

    Dim strTemp As String

    strTemp = Mid$(strPath, InStrRev(strPath, ")") + 1)

    FileNameNoExt = Left$(strTemp, InStrRev(strTemp, ")") - 1)

End Function
'Find all files in a directory

Private Function FindFiles(ByVal path As String, ByVal ext As String, ByRef files() As String) As Boolean

    Dim ffile As String

    ffile = Dir$(path & "\*." & ext)

    Do

        If (ffile <> vbNullString) Then

            If (FindFiles = False) Then

                ReDim files(0) As String

                FindFiles = True

            Else

                ReDim Preserve files(UBound(files) + 1) As String

            End If

        files(UBound(files)) = ffile

        ffile = Dir

        Else

End If
Exit Do

End If

Loop Until (ffile = vbNullString)

End Function

' Let the user browse for a directory. Return the
' selected directory. Return an empty string if
' the user cancels.

Public Function BrowseForDirectory() As String

Dim browse_info As BrowseInfo

Dim item As Long

Dim dir_name As String

' modified for MS Access/VBA

With browse_info

' .hwndOwner = Application.hWndAccessApp

.pidlRoot = 0

.sDisplayName = Space$(260)
.sTitle = "Select Directory"

.ulFlags = 1 ' Return directory name.

.lpfn = 0

.lpParam = 0

.iImage = 0

End With

item = SHBrowseForFolder(browse_info)

If item Then

    dir_name = Space$(260)

    If SHGetPathFromIDList(item, dir_name) Then

        BrowseForDirectory = Left(dir_name, InStr(dir_name, Chr$(0)) - 1)

    Else

        BrowseForDirectory = ""

    End If

End If
End Function

'Determine if a file exists already

Function FileThere(FileName As String) As Boolean
    FileThere = (Dir(FileName) > "")
End Function

'This module is then run against the final data file to calculate the final data for use

'Requirements are that the data is sorted by hour, genotype and sex first, otherwise this will not work

Sub CalculateFinalData()
    Sheets("Sheet2").Name = "ConsolidatedData"
    Sheets("ConsolidatedData").Select
    Range("A1").Value = "Temperature"
    Range("A2").Value = "Hour"
    Range("B2").Value = "F Control"
    Range("C2").Value = "M Control"
<table>
<thead>
<tr>
<th>Range (cell)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2</td>
<td>&quot;F Midi&quot;</td>
</tr>
<tr>
<td>E2</td>
<td>&quot;M Midi&quot;</td>
</tr>
<tr>
<td>F1</td>
<td>&quot;DTcm&quot;</td>
</tr>
<tr>
<td>F2</td>
<td>&quot;F Control&quot;</td>
</tr>
<tr>
<td>G2</td>
<td>&quot;M Control&quot;</td>
</tr>
<tr>
<td>H2</td>
<td>&quot;F Midi&quot;</td>
</tr>
<tr>
<td>I2</td>
<td>&quot;M Midi&quot;</td>
</tr>
<tr>
<td>J1</td>
<td>&quot;RQ&quot;</td>
</tr>
<tr>
<td>J2</td>
<td>&quot;F Control&quot;</td>
</tr>
<tr>
<td>K2</td>
<td>&quot;M Control&quot;</td>
</tr>
<tr>
<td>L2</td>
<td>&quot;F Midi&quot;</td>
</tr>
<tr>
<td>M2</td>
<td>&quot;M Midi&quot;</td>
</tr>
<tr>
<td>N1</td>
<td>&quot;kcal per RQ&quot;</td>
</tr>
<tr>
<td>N2</td>
<td>&quot;F Control&quot;</td>
</tr>
<tr>
<td>O2</td>
<td>&quot;M Control&quot;</td>
</tr>
<tr>
<td>P2</td>
<td>&quot;F Midi&quot;</td>
</tr>
</tbody>
</table>
Range("Q2").Value = "M Midi"

Range("R1").Value = "kcal per min"

Range("R2").Value = "F Control"

Range("S2").Value = "M Control"

Range("T2").Value = "F Midi"

Range("U2").Value = "M Midi"

Range("V1").Value = "MR"

Range("V2").Value = "F Control"

Range("W2").Value = "M Control"

Range("X2").Value = "F Midi"

Range("Y2").Value = "M Midi"

Sheets("CompleteData").Select

Dim rowCount As Integer

rowCount = 3

Dim startPos As Integer, endPos As Integer

Dim colPos As Integer
startPos = 3

Dim currentSex As String, sex As String, currentGenotype As String, genotype As String

Dim currentHour As Integer, hour As Integer

hour = -1

'Go through each row, determine the sex, genotype and hour, when one changes we have a block that can then be calculated

Do While Range("A" & rowCount).Value <> ""

If sex = "" Then

    sex = Range("B" & rowCount).Value

End If

If genotype = "" Then

    genotype = Range("C" & rowCount).Value

End If

If hour = -1 Then

    hour = Range("H" & rowCount).Value

End If
End If

currentSex = Range("B" & rowCount).Value

currentGenotype = Range("C" & rowCount).Value

currentHour = Range("H" & rowCount).Value

If currentGenotype <> genotype Then

    Call CalculateColumn(sex, genotype, startPos, endPos, hour + 2)

    startPos = endPos + 1

    genotype = currentGenotype

If currentSex <> sex Then

    sex = currentSex

    End If

ElseIf currentSex <> sex Then

    Call CalculateColumn(sex, genotype, startPos, endPos, hour + 2)

    startPos = endPos + 1

    sex = currentSex

    If currentGenotype <> genotype Then
genotype = currentGenotype

End If

End If

If currentHour <> hour Then

    Range("ConsolidatedData!A" & hour + 2).Value = hour

    hour = currentHour

End If

endPos = rowCount

rowCount = rowCount + 1

Loop

Call CalculateColumn(sex, genotype, startPos, endPos, hour + 2)

Range("ConsolidatedData!A" & hour + 2).Value = hour

End Sub

'Calculate the column of data depending on the criteria

Sub CalculateColumn(sex As String, genotype As String, startPos As Integer, endPos As Integer, hour As Integer)
If sex = "F" And genotype = "control" Then

    Range("ConsolidatedData!B" & hour).Value =
        "=AVERAGE(CompleteData!Z" & startPos & ":Z" & endPos & ")" 'Temp

    Range("ConsolidatedData!F" & hour).Value =
        "=AVERAGE(CompleteData!R" & startPos & ":R" & endPos & ")" 'DTcm

    Range("ConsolidatedData!J" & hour).Value =
        "=AVERAGE(CompleteData!N" & startPos & ":N" & endPos & ")" 'RQ

    Range("ConsolidatedData!N" & hour).Value =
        "=AVERAGE(CompleteData!O" & startPos & ":O" & endPos & ")" 'kcal per RQ

    Range("ConsolidatedData!R" & hour).Value =
        "=AVERAGE(CompleteData!P" & startPos & ":P" & endPos & ")" 'kcal per min

    Range("ConsolidatedData!V" & hour).Value =
        "=AVERAGE(CompleteData!Q" & startPos & ":Q" & endPos & ")" 'MR

ElseIf sex = "F" And genotype = "midi" Then

    Range("ConsolidatedData!D" & hour).Value =
        "=AVERAGE(CompleteData!Z" & startPos & ":Z" & endPos & ")" 'Temp

    Range("ConsolidatedData!H" & hour).Value =
        "=AVERAGE(CompleteData!R" & startPos & ":R" & endPos & ")" 'DTcm
Range("ConsolidatedData!L" & hour).Value = 
"=AVERAGE(CompleteData!N" & startPos & ":N" & endPos & ")" 'RQ

Range("ConsolidatedData!P" & hour).Value = 
"=AVERAGE(CompleteData!O" & startPos & ":O" & endPos & ")" 'kcal per RQ

Range("ConsolidatedData!T" & hour).Value = 
"=AVERAGE(CompleteData!P" & startPos & ":P" & endPos & ")" 'kcal per min

Range("ConsolidatedData!X" & hour).Value = 
"=AVERAGE(CompleteData!Q" & startPos & ":Q" & endPos & ")" 'MR

ElseIf sex = "M" And genotype = "control" Then

Range("ConsolidatedData!C" & hour).Value = 
"=AVERAGE(CompleteData!Z" & startPos & ":Z" & endPos & ")" 'Temp

Range("ConsolidatedData!G" & hour).Value = 
"=AVERAGE(CompleteData!R" & startPos & ":R" & endPos & ")" 'DTcm

Range("ConsolidatedData!K" & hour).Value = 
"=AVERAGE(CompleteData!N" & startPos & ":N" & endPos & ")" 'RQ

Range("ConsolidatedData!O" & hour).Value = 
"=AVERAGE(CompleteData!O" & startPos & ":O" & endPos & ")" 'kcal per RQ

Range("ConsolidatedData!S" & hour).Value = 
"=AVERAGE(CompleteData!P" & startPos & ":P" & endPos & ")" 'kcal per min
Range("ConsolidatedData!W" & hour).Value =
"=AVERAGE(CompleteData!Q" & startPos & ":Q" & endPos & ")" 'MR

ElseIf sex = "M" And genotype = "midi" Then

Range("ConsolidatedData!E" & hour).Value =
"=AVERAGE(CompleteData!Z" & startPos & ":Z" & endPos & ")" 'Temp

Range("ConsolidatedData!I" & hour).Value =
"=AVERAGE(CompleteData!R" & startPos & ":R" & endPos & ")" 'DTcm

Range("ConsolidatedData!M" & hour).Value =
"=AVERAGE(CompleteData!N" & startPos & ":N" & endPos & ")" 'RQ

Range("ConsolidatedData!Q" & hour).Value =
"=AVERAGE(CompleteData!O" & startPos & ":O" & endPos & ")" 'kcal per RQ

Range("ConsolidatedData!U" & hour).Value =
"=AVERAGE(CompleteData!P" & startPos & ":P" & endPos & ")" 'kcal per min

Range("ConsolidatedData!Y" & hour).Value =
"=AVERAGE(CompleteData!Q" & startPos & ":Q" & endPos & ")" 'MR

End If

End Sub
VITA

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- 2010, Ph.D. in Physiology, The Pennsylvania State University, USA
- 2004, M.S. in Animal Sciences, The Pennsylvania State University, USA
- 2002, B.S. in Animal Sciences, The Pennsylvania State University, USA

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- 2010 Huck Institute Scholarship, The Pennsylvania State University
- 2004-2005, University Graduate Fellowship, The Graduate School & Intercollege Graduate Degree Program in Physiology, The Pennsylvania State University
- 2003 High Point Individual, Advanced, Returning Student Category, Animal Behavior and Welfare Competition, Michigan State University
- 1996 - 2001 Dolphin Scholarship Foundation Scholarship, Virginia Beach, VA
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- 1996 Fleet Reserve Association Scholarship, Alexandria, VA

Research Interests:

- Investigation of stress resistance by a variety of physiological techniques
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- Craniofacial stenosis

Publications

