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Age-Related Changes in two Muscle Enzymes of the Hawk Moth

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AGE -RELATED CHANGES IN TWO MUSCLE ENZYMES OF THE HAWK MOTH,

MANDUCA SEXTA

by

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to examine the thesis of Connor McMahon

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ABSTRACT

AGE -RELATED CHANGES IN TWO MUSCLE ENZYMES OF THE HAWK MOTH,

MANDUCA SEXTA

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Aging has been associated with significant declines in muscle strength and mass. The specific molecular mechanisms behind these changes, however, are not well known. One potential mechanism behind muscle aging could be enzymatic dysfunction. This research examines the enzyme changes that occur in the dorsolateral flight muscles of the hawk moth. The hawk moth, *Manduca sexta,* is an excellent model of muscle aging due to its short lifespan, ease of rearing, and endothermic flight muscles. We assayed citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (HADH) to determine the activity of the tricarboxylic acid cycle and fatty acid oxidation, respectively. A significant change in HADH activity was observed in male hawk moths between daytime and nighttime, but this change was not detected in females. Significant changes in CS activity occurred between middle aged and aged female hawk moths. Overall, CS activity increased with age for females, and aged females showed less distinction between daytime and nighttime CS activity. Increases in CS activity suggest age-related TCA cycle dysfunction in female hawk moths. This research identifies how beta oxidation and TCA activity change differently for male and female hawk moths via aging.

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CHAPTER ONE

Introduction

Biological aging is a natural process of life that results in a variety of physical ailments that can significantly reduce health and longevity. While not as dramatic as other age-related disorders like type II diabetes, cardiovascular disease, or neuro-degenerative disease, the gradual decline in muscle function associated with aging can have a significant impact on the physical and psychological health of an aged individual. Campisi (2013) reports that the senescence process compromises tissue regeneration and molecular repair mechanisms, lessening a body's ability to fight infection and replace damaged tissue. Muscle senescence significantly reduces muscle mass and function and can increase the likelihood of morbidity or mortality for the aging individual (Childs et al., 2015). Additionally, the ability for muscle tissue to respond to physical demands decreases as organisms age. In humans, losing physical independence can be a demoralizing experience for aging individuals (Rasmussen et al., 2006), so reducing the magnitude of sarcopenia (the loss of muscle tissue as a characteristic part of biological aging) is an important advancement for an aging world population. Understanding the physiological mechanisms behind sarcopenia is a vital step in developing therapeutic interventions to curb the effects of age-related muscle dysfunctions.

Although sarcopenia is likely a multifactorial process, metabolic changes have been suggested as critical factors causing the loss of muscle function (Barzilai et al., 2012). Muscles require adequate energy to function and maintain their mass, so major metabolic changes can alter muscle tissue regeneration and repair. Muscle aging is

marked by increased dysregulation of mitochondrial biogenesis and loss of metabolic efficiency (Yuho et al., 2017). Decreased mitochondrial vitality is associated with reactive oxygen species (ROS) accumulation, causing cellular damage (Short et al., 2005). This process can deeply reduce muscle maintenance and could contribute to sarcopenia.

Various models of muscle aging can provide valuable insight into age-related metabolic changes, and each model offers unique advantages and disadvantages. Rhesus macaques (*Macaca mulatta*) have a high genetic similarity to humans, and their relatively large muscle size aids muscle sampling (Uno, 1997). The shortcoming with these and other mammalian models is the time and resources it costs for the organism to rear and age. While being less genetically similar to humans, invertebrate models can provide important insights into sarcopenia with a lower cost of rearing and less time to invest in aging. Additionally, sarcopenia occurs more markedly in short-lived species such as *Drosophila melanogaster* (Demontis et al., 2013). Insect models, being smaller than mammalian models, offer less muscle tissue per organism but it becomes much easier to raise many test subjects and several generations over a short time. *Manduca sexta* offers the advantages of invertebrate models (short-lifespan, low-cost, and more-dynamic sarcopenia-like response) while its endothermic dorsolateral flight muscles are similar in physiology to vertebrate models. This research examines the enzymatic changes in two key muscle enzymes that occur in aging *Manduca sexta* flight muscles in order to better understand the mechanisms behind muscle aging and perhaps sarcopenia.

Background

Research in the field of aging not only focuses on lengthening lifespan, but considerable effort also goes into increasing healthspan. Medically, improving the quality of late-life physiology requires understanding how aging damages the body. Normal aging causes the accumulation of mutations in DNA, some of which affect mitochondrial efficiency and overall metabolic efficiency (Houtkooper et al., 2011). When the genes that control metabolism are altered, the ability for muscle tissue to respond to the energy demands of physical activity can be diminished. Wone et al. (2018a) described a shift in metabolite concentrations showing an increase in metabolites associated with glycolysis and the tricarboxylic acid (TCA) cycle and a decrease in fatty acids associated with β-oxidation in the aged dorsolateral flight muscles of *M. sexta*. Reductions in fatty acid oxidation intermediates have also been observed in brown Norway rats and C57BL/6J mice (Tucker & Turcotte, 2002; Houtkooper et al., 2011). Age-related decreases in TCA activity have been noted in *Drosophila* (Girardot et al., 2006; Demontis et al., 2013). These findings suggest that aging muscle is associated with changes in glycolysis, TCA activity, and fatty acid demand. Determining how the mechanics of these energy pathways are affected by aging could be a key element in understanding the physiological mechanisms of sarcopenia.

Aging influences the oxidative capacity of skeletal muscle. In *Caenorhabditis elegans*, phosphoenolpyruvate carboxykinase activity decreases with age while pyruvate kinase activity increases with age (Feng et al., 2016). These enzymatic changes shift energy away from oxidative energy pathways towards increased anaerobic glycolysis.

Age-related metabolic protein damage is more common in aerobic fiber types than in glycolytic fiber types in male C57BL/6 mice (Choksi et al., 2008). Skeletal muscle aging is associated with increased glycolytic activity and impairments in glycogen synthesis and glucose uptake into skeletal muscle cells (Barzilai et al., 2012; Houtkooper et al., 2011). In humans, insulin sensitivity declines as the result of metabolic syndrome and abdominal obesity is commonly observed as a physical manifestation of metabolic decline (Huffman & Barzilai, 2009; Rasmussen et al., 2006). Insulin resistance is associated with an inability to maintain glucose levels in blood and within muscle cells. Current literature suggests that anaerobic activity increases to account for the functional decline of aerobic metabolism.

Muscular nicotinamide adenine dinucleotide (NAD^+) declines as a function of age in humans and model organisms alike. Evidence suggests that a drop in NAD^+ levels diminishes the activity of sirtuin, a vital protein regulator of cellular health and a key protein in aging (Mouchiroud, 2013). This family of proteins activates enzymes involved in fatty acid oxidation and the TCA cycle (Hirschey & Zhao, 2015). Sirtuin is only active in the presence of NAD^+, so declines in fatty acid oxidation or similar metabolic processes that increase cellular NAD^+ can contribute to the functional decline in the activity of aged cells. Genetic or pharmacological interventions that promote NAD^+ production have been shown to promote longevity and healthspan in *C. elegans* (Mouchiroud, 2013). Because NAD^+ levels decline with age, metabolic pathways that regulate cellular and extracellular NAD^+, such as fatty acid oxidation, might decline as the result of aging.

During fatty acid oxidation, triglycerides are broken down for energy. Skeletal muscle can account for a significant portion of total fatty acid oxidation, over 50% of a human body's total fatty acid oxidation may occur in muscle tissue (Tucker & Turcotte, 2002). In brown Norway rats, middle aged and advanced aged specimens show lower overall oxidation than young rats, suggesting that the resting ability for aged rats to oxidize fatty acids is decreased (Tucker & Turcotte, 2002). In insects such as *M. sexta*, triglyceride oxidation is a vital source of water, producing nearly double the metabolic water return compared glycogen breakdown (Arrese & Soulages, 2010). Additionally, aging flight muscle accumulates fatty acids, further suggesting that fatty acid oxidation is decreased (Houtkooper et al., 2011; Wone et al., 2018a). Extracellular fatty acids also accumulate through aging in humans, as fatty acids become more abundant in plasma when a person reached middle and advanced age (Lawton et al., 2008). Current research suggests that overall fatty acid oxidation declines as part of muscle senescence.

Citrate synthase (CS) is a rate-limiting enzyme that catalyzes in the first step of the TCA cycle. This protein is an important indicator of the oxidative capacity of mitochondria (Short et al., 2005). Biopsies of the vastus lateralis from healthy humans have determined that the content and activity of CS declines in the muscle mitochondria as a person ages (Short et al., 2005). Additionally, research by Rooyackers et al. (1996) suggests a decline in the rate of translation of CS mRNAs in aged human muscle tissue. This decline in CS activity is not consistent between different human muscle groups, however, as certain muscle studies show no relationship between age and CS activity (Houmard et al., 1998). In *M. sexta*, metabolomics data suggests an increase in CS activity for aging female hawk moths with little change for male hawk moths (Wone et

al., 2018a). In general, the current understanding of aging trends argues that there is an overall reduction of mitochondrial efficiency and mitochondrial biogenesis (Barzilai et al., 2012). There is some disagreement in whether CS activity declines account for the reduction in mitochondrial efficiency, and variability in activity changes between vertebrate muscles (Houmard et al., 1998) makes general conclusions difficult to discern.

CHAPTER TWO

Methodology

Experimental Animals

Wone et al. (2018a) established the hawk moth, *M. sexta*, as a complementary invertebrate model animal for examining muscle senescence. Due to their short lifespan (days to weeks), the effects of aging can be readily observed and studied across the entire course of aging. *Manduca sexta* is a useful model of vertebrate muscle aging because of the endothermic nature of their flight muscles (George & Daniel, 2011). Hawk moths must vibrate their wings to warm their flight muscles to ~39ºC for efficient flight (George & Daniel, 2011). The flight muscles of *M. sexta* are synchronous, meaning the muscle fibers contract once per every nerve impulse (Yuan et al., 2015). Because synchronous flight muscles of invertebrates are highly-active and inherently energy-inefficient, the majority of chemical energy used for flight (90-95%) is released as heat rather than converted to mechanical energy (Josephson & Stevenson, 1991). Studying this invertebrate model is useful in determining molecular processes that contribute to muscle senescence, which could further be translated to mammalian and human studies.

Metabolism is intimately linked with the circadian clock, as changes in lipid storage, insulin activity, and glucose metabolism occur in muscle cells according to temporal positioning in both humans and hawk moths (Dyar et al., 2018). Diel time may be a particularly important factor in metabolic activity for *M. sexta*, as hawk moths are nocturnal animals and metabolically most active at night. Circadian rhythmicity is a key regulator of skeletal muscle metabolism. In *Bmal1* mice, the majority of circadian

mRNAs in skeletal muscle were determined to have metabolic roles (Harfmann et al., 2016). The diel time-specific regulation of metabolism is important for anticipating activity states and can greatly influence the uptake and metabolism of glucose into skeletal muscle tissue. Diel time is a key variable examined in these assays.

Additionally, important variables in metabolic activity are sex and age. These two variables are intimately linked, as sex is a significant determinant of the lifespan of humans as well as model organisms such as *M. sexta* (Wone et al., 2018a). For the purposes of this study, age categories post-eclosion were determined for middle aged (Male = 2 days; Female = 4 days) and aged (Male = 5 days; Female = 7 days) specific to each sex (Wone et al., 2018a). This study used the half of the measured lifespan for males (fed = 6.1 days; unfed = 4.9 days) and females (fed = 10.9 days; unfed = 8.0 days) as a marker for middle age (Wone et al., 2018a). Half of all tissues sampled from *M. sexta* were taken during the day time, while the other half were taken at night time to equally represent both diel times in the assays. Beginning at middle age, adult moths were euthanized by decapitation, and their dorsolateral flight muscle samples were obtained via dissection and flash frozen in liquid nitrogen. Muscle collection for day samples began at 0900 h and night samples began at 2100 h (following one hour of activity). Less than 90 seconds passed between time of death and flash freezing of the extracted muscle tissue samples. The age at the time of death was individually recorded. Tissue samples were then ground under liquid nitrogen with a mortar and pestle and stored at -80 °C until being homogenized 48 hours prior to the assays being run.

Enzyme Spectrophotometric Assays

The activities of citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (HADH) were determined via photometric analysis of the rate of absorption change for each selected enzyme. Photometric protein assays are useful in measuring the quantity of specific metabolites, and effective assays allow the observation of a single enzyme's activity in a tissue sample (Choksi et al., 2008). Groups of male and female flight muscle samples (n=20/sex) were assayed on separate Eppendorf® Microplate UV-VIS, 96/F plates (Eppendorf, Hauppauge, NY). Each plate consisted of four quadrants divided into age and diel time categories. Twenty milligrams of each flight muscle sample were homogenized with 200μl (1:10 ratio) of ice-cold homogenizing buffer (100 mM phosphate, 5 mM EDTA, 0.1% Triton x-100, pH 7.2) before beginning the assays. This process was used to prepare and organize muscle samples for both the citrate synthase and β-hydroxyacyl-CoA dehydrogenase assays. Test groups were divided evenly between sex, age, and diel time. Each test group had three technical replicates and five biological replicates (e.g. five middle-aged male samples taken during the day, each with three technical replicates). All assays were run within a period 48 hrs following the time of muscle tissue homogenization.

As stated previously, CS activity was used to represent overall tricarboxylic acid cycle (TCA) activity. As this TCA reaction takes place, acetyl-CoA is catalyzed by CS into reduced form CoA-SH. In the final reaction mixture, the thiol group of CoA-SH further reacts with 5,5`-dithiobis [2-nitrobenzoic acid] (DTNB) in the reaction mixture to form the yellow product 5-thio-2-nitrobenzoic acid (TNB). TNB is observed spectrophotometrically by measuring absorbance at 412 nm. The rate in which TNB

concentration increased was used to determine CS activity. The reaction mixture for the citrate synthase assay is 100 mM Tris-HCl, 5 mM EDTA, 0.22 mM Acetyl-CoA, 0.5 mM oxaloacetate, and 0.1 mM DNTB at a pH of 8.0.

For the fatty acid oxidation reaction assay, HADH catalyzes the oxidation of β-Nicotinamide Adenine Dinucleotide (NADH) into β-Nicotinamide Adenine Dinucleotide, (NAD^+). As the reaction progresses, the amount of NADH per well should decrease. The presence NADH is observed spectrophotometrically at 340 nm, and the rate of loss of NADH was used to determine the activity of HADH. The reaction mixture for the β-Hydroxyacyl-CoA Dehydrogenase (HADH) assay contained 100 mM Triethanolamine-HCl, 5 mM EDTA, 0.1 mM Acetoacetyl-CoA, 0.28 mM NADH at a pH of 7.0.

Two microlitres of tissue homogenate were added to the reaction mixture of each well before the final volume was brought to 200 μl. Absorbance was read on a kinetic loop at 39 °C for 15 min with an accuSkan™ GO UV/Vis Microplate Spectrophotometer, Fisherbrand™ model #14-377-579 (Thermo Fischer Scientific, Pittsburgh, PA). The kinetic loop was run for 90 loops at a 10 sec interval between well readings. Data were compiled in SkanIt Software™ for Microplate Readers ver. 4.1.0.43 (Thermo Scientific, Waltham, MA).

Quantitative Analysis

The CS activity (U/mL) was calculated using the following equation:

$$\text{U/ml enzyme} = \frac{V \times dil}{\varepsilon \times d \times a} \times A_{412},$$

where: V (ml) = Total volume in well (= 200 μl or 0.2 ml), ε (mM^{-1} cm^{-1}) = millimolar extinction coefficient for TNB at 412 nm {mmol/L} (= 13.6), d (cm) = light path of the well (= 0.67 cm), a (ml) = volume of diluted homogenate (= 0.002 ml), ΔA_{412} = sample measurement (A$_{412nm}$/min) – background measurement (A$_{412nm}$/min), dil = dilution factor of homogenate (= 11). Unit (U) definition - One unit will convert 1.0 μmole of DTNB to TNB per minute at pH 8.0 at 39° C.

The HADH activity (U/mL) was calculated using the following equation:

$$\text{U/ml enzyme} = \frac{V \times dil}{\varepsilon \times d \times a} \times A_{340},$$

where V (ml) = Total volume in well (= 200 μl or 0.2 ml), ε (mM^{-1} cm^{-1}) = millimolar extinction coefficient for NADH at 340nm {mmol/L} (= 6.22), d (cm) = light path of the well (= 0.67 cm), a (ml) = volume of diluted homogenate (=0.002 ml), ΔA_{340} = sample measurement (A$_{340nm}$/min) – background measurement (A$_{412nm}$/min), dil = dilution factor of homogenate (= 11). Unit (U) definition - One unit will convert 1.0 μmole of acetoacetyl-CoA to β-hydroxybutyryl-CoA per minute at pH 7.0 at 39° C in the presence of β-NADH.

Statistics

From the CS and HADH assays, kinetic absorbance data were compared between age, sex, and diel time. The average activity of the three technical replicates from each tissue sample was used. RStudio® (RStudio, Boston, MA) was used analyzed the data. Three-way analysis of variance (ANOVA) tests were performed to determine the effects of sex, diel time, and age on CS and HADH activity, respectively. Separate ANOVAs provided data on the interaction of the three independent factors (age, sex, and diel time) on the dependent factor (enzyme activity). All significant results were analyzed *post hoc* via Tukey Test to determine which sample groups significantly differed. Significance was determined with an α level of $P \leq 0.05$.

CHAPTER THREE

Results

HADH Activity

An overall ANOVA indicated significant difference in HADH activity between sexes ($F_{1,38} = 0.128$; $P = 0.723$). A three-way ANOVA revealed a significant difference in HADH activity between male samples taken at day time (n = 10) and male samples taken at night time (n = 10) (Figure 1). HADH activity for night samples increased by 26.8% on average when compared to day samples from male hawk moths ($F_{1,19} = 6.967$; $P = 0.020$). Unlike male samples, there was no significant difference for the HADH activity of female samples taken at different diel time $F_{1,19} = 0.374$; $P = 0.549$). Further, three-way ANOVA indicated no significant difference in HADH activity between age groups for male ($F_{1,19} = 0.932$; $P = 0.349$) or female ($F_{1,19} = 0.374$; $P = 0.549$) hawk moths (Table 1).

Table 1: Means and standard deviation for each assay group. MA - middle aged, A - aged, F – female, and M – male.

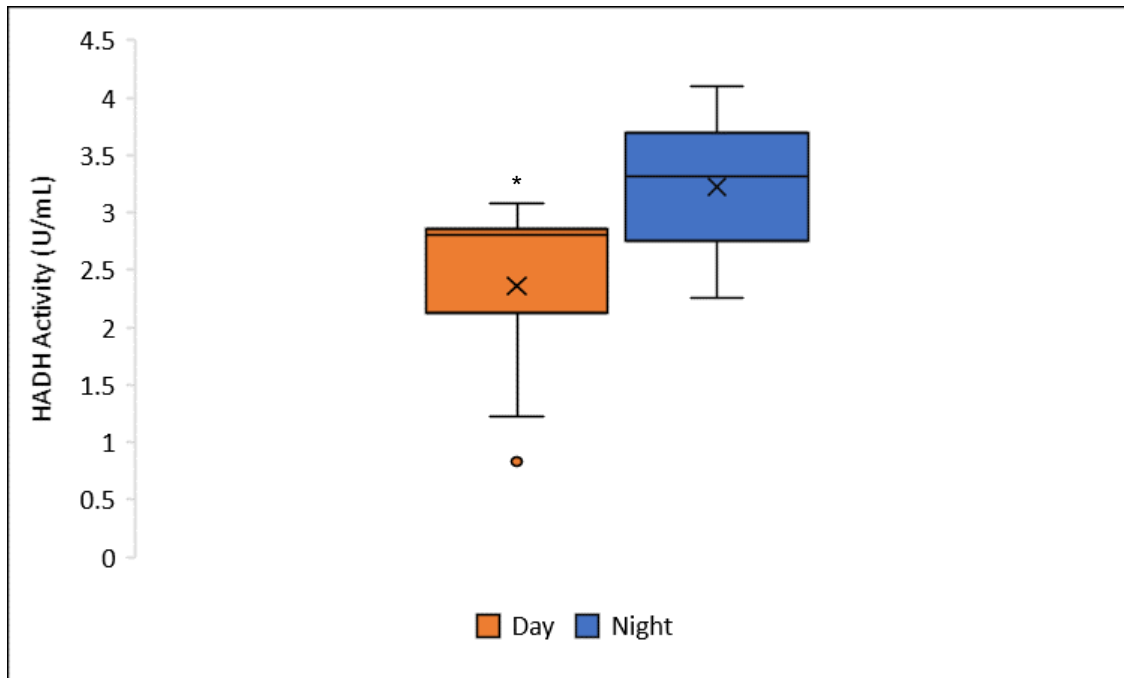| Sample Group	Mean (CS)	SE (CS)	Mean (HADH)	SE (HADH)
F-MA	20.825	8.347	2.824	1.073
F-A	26.766	3.909	2.589	0.636
M-MA	27.410	5.094	2.952	0.885
M-A	27.639	4.196	2.636	0.766

Figure 1: Mean HADH activity for male samples separated by diel time. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data, respectively. Circles represent outlying data points, x = the mean, and the line = the median. Unit (U) definition - One unit will convert 1.0 μmole of acetoacetyl-CoA to β-hydroxybutyryl-CoA per minute at pH 7.0 at 39 °C in the presence of β-NADH. Single asterisks (*) denote P < 0.05.

CS Activity

An overall ANOVA indicated significant difference in CS activity between sexes ($F_{1,38}$ = 4.454; P = 0.042). Citrate synthase activity did not vary significantly by diel time for male groups ($F_{1,18}$ = 0.518; P = 0.482) and was almost significantly different for female groups ($F_{1,18}$ = 4.248; P = 0.055, Figure 2). There was no significant change in the CS activity between male age groups ($F_{1,18}$ = 0.011; P = 0.917). The three-way ANOVA indicated a significant difference in mean CS activity between middle aged and aged female samples (Figure 3). Specifically, the CS activity of middle-aged females (n = 10, mean = 24.12) was significantly lower than that of aged females (n = 10, mean = 27.20).
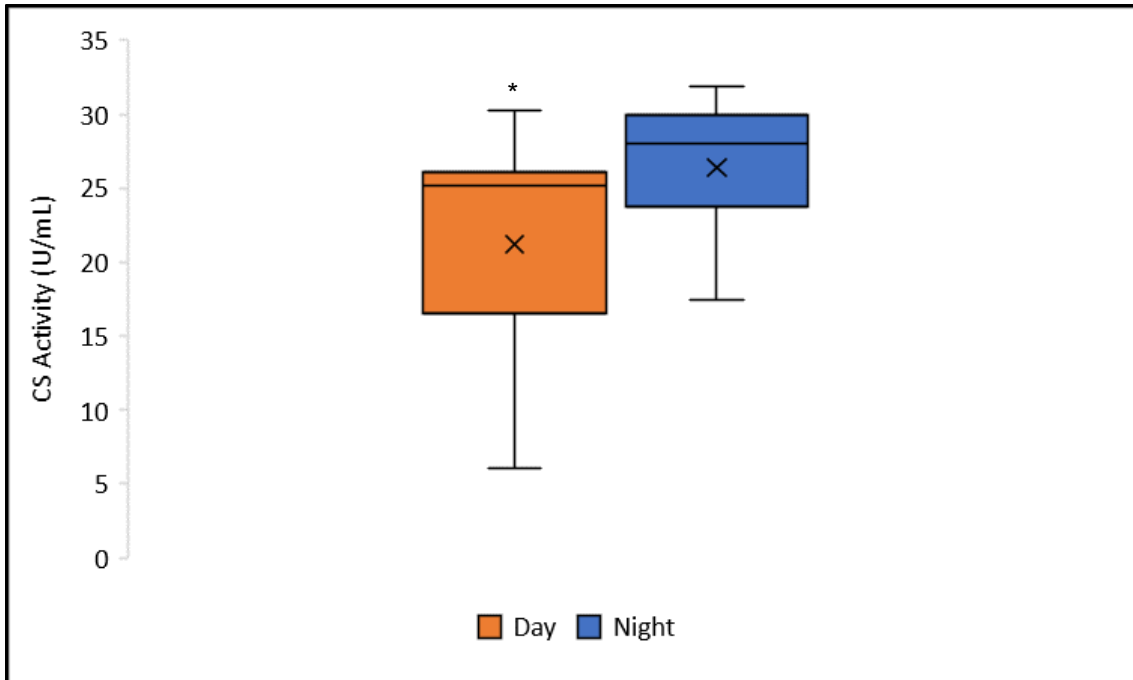
Figure 2: Mean CS activity for female samples separated by diel time. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data, respectively. Circles represent outlying data points, x = the mean, and the line = the median. Unit (U) definition - One unit will convert 1.0 µmole of DTNB to TNB per minute at pH 8.0 at 39 °C. Single asterisks (*) denote P < 0.05.
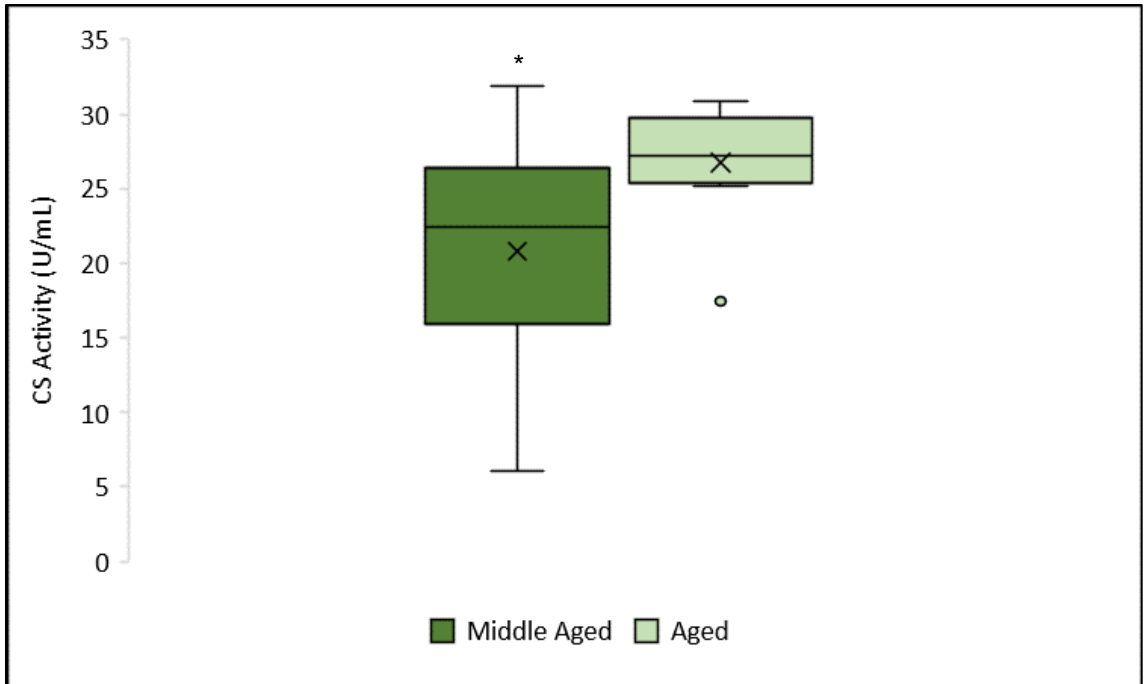
Figure 3: Mean CS activity for female samples separated by age group. The y-axis plots represent the activity mean (×) and median (−) values for upper and lower quartiles, respectively. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data, respectively. Circles represent outlying data points, x = the mean, and the line = the median. Unit (U) definition - One unit will convert 1.0 μmole of DTNB to TNB per minute at pH 8.0 at 39° C. Single asterisks (*) denote $P < 0.05$.

Further analyzing the significance of these results, a *post hoc* Tukey test identified which diel time and age groups varied significantly from one another. CS activity significantly varied between middle aged day and aged day female samples. CS activity also significantly varied between middle aged day and aged night female samples. The Tukey comparison between middle aged day and middle aged night female samples yielded nearly-significant change ($P = 0.053$). Results of the Tukey comparison for female samples are listed in Table 1. A visual comparison of the mean CS activity for each of the significantly varying experimental groups identified by the Tukey test are displayed in Figure 4.

Table 2: Results of Tukey Test for female samples. Difference refers to Fischer's least significant difference. MA - middle aged, A – aged. Adjusted *P* is calculated per Tukey test. Single asterisks (*) denote P < 0.05.

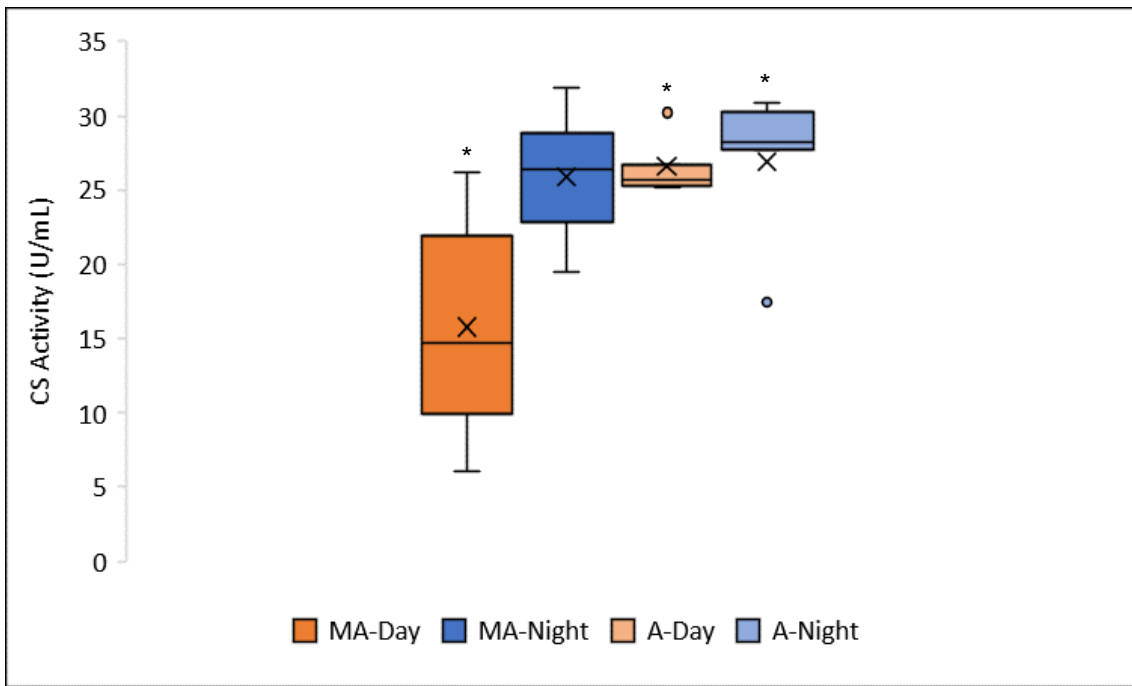| Paired Comparison	Difference	Adjusted *P*
MA:Day-A:Day	-10.830	0.036*
A:Night-A:Day	0.313	1.000
MA:Night-A:Day	11.143	0.030 *
MA:Night-MA:Day	10.092	0.053
MA:Night-A:Night	-1.051	0.991

Figure 4: Mean CS activity for female samples separated by diel time and age groups. The y-axis plots represent the activity mean (×) and median (−) values for upper and lower quartiles, respectively. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data, respectively. Circles represent outlying data points, x = the mean, and the line = the median. Age groups are denoted by "MA" (middle aged) and "A" (aged). Unit (U) definition - One unit will convert 1.0 μmole of DTNB to TNB per minute at pH 8.0 at 39° C. Single asterisks (*) denote P < 0.05. MA-day to MA-night adjusted P ≤ 0.0533. MA-day A-day adjusted P ≤ 0.0357. MA-day to A-night adjusted P ≤ 0.0301.

CHAPTER FOUR

Discussion

HADH activity for male hawk moths was increased during nighttime compared to daytime. Male hawk moths do not typically feed during night active periods when provided with the same food as females and are therefore unable to obtain new fatty acids for metabolism (Arrese, 1999). Increased beta oxidation at nighttime might reflect the need for male hawk moths to utilize stored fatty acids to meet energy demands during active periods. The findings of Wone et al. (2018a) described how the presence of a large number of lysolipids (i.e., behenate, margarate, oleate/vaccenate, palmitate, and stearate) decreased with age in males, reflecting the use of stored larval lipids to meet the energy demands. The loss of lysolipids with age may also support the fact that male hawk moths utilized stored fatty acids instead of feeding during active periods. Additionally, lowering HADH activity during inactive hours might be a mechanism by which male hawk moths employ to conserve a limited supply of stored fatty acids.

Compared to male HADH activity changes, female samples did not show a significant difference in HADH activity between day and night diel time. Female moths are more likely to feed during active hours, so the sex differences in day/night variation of HADH activity could be attributed to the feeding habits of male and female hawk moths (Arrese, 1999). One of the reasons females typically live longer than males might be that females can obtain new fatty acids instead of exhausting a stored supply of larval fatty acids like males (Levin et al., 2017). These data reinforce the notion that adult female hawk moths feed while adult male hawk moths do not feed. Females may need to

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acquire energy from sources other than stored fatty acids to produce and grow eggs while sustaining energy to survive until they able to lay eggs. The hawk moths in this experiment were fed artificial nectar (i.e. sugar water), so females would have an energy supply of glucose instead of relying on the breakdown of fatty acids. Alternatively, males may rely more on the fatty acids stored at larval stages than females, potentially explaining why changes in HADH activity between diel time are seen in male samples and not female samples.

The results the CS assays are consistent with the findings of Wone et al. (2018a). Previous work found that aging female hawk moths tended to show a 3.09-fold increase in the abundances of TCA cycle metabolites, such as citrate and isocitrate, in aging hawk moths (Wone et al., 2018a). The increased presence of these metabolites could suggest a greater energy demand with age. These results might differ from mammalian studies because of the difference in lifespan between species. Since hawk moths live short lives than mammals, they may experience different enzymatic changes specific to their longevity. Alternatively, these changes could be indicative of metabolic dysfunction. Dysregulation of TCA activity is linked to mitochondrial dysfunction and insulin resistance in both rodent and human subjects, corresponding to a number of pathologies, such as diabetes and fatty liver (Satapati et al., 2012; Huffman & Barzilai, 2009; Rasmussen et al., 2006). Because aged females do not demonstrate a measurable difference in CS activity between active and inactive periods like middle-aged females, aged females may not be able to regulate TCA activity per their day/night energy demands.

Diel time had a significant effect on CS activity for female adult hawk moths. Middle aged samples taken during the day had the lowest CS activity out of any of the female sample groups. Physically, this may reflect the lower energy demands of the inactive day period for *M. sexta*. Because the daytime CS activity is lower in middle aged females than aged females, the ability to modulate the TCA cycle during inactive periods may be reduced by the aging process. This consideration may be reinforced by the lack of change between the day and night CS activities for aged females. These data might indicate that aged females are less able to alter TCA activity to accommodate changes in energy demands or that the energy demands are higher in both day and night for aged females. Metabolic inflexibility could be a considerable aspect of muscle aging for female hawk moths. In future research, larger sample size could increase the power of these assays and clarify differences measured in CS activities between female diel time groups.

The sex differences in age-related changes in CS activity might be related to lifespan. Because males have a shorter lifespan than females, male hawk moths could die prior to substantial age-related changes in TCA activity. TCA dysregulation may be restricted to very late ages in hawk moths, so males may not live long enough to experience CS enzyme changes. Alternatively, the increase in CS enzyme function with age in females may be a mechanism to cope with metabolic aging. Increasing TCA activity could be a means of providing adequate cellular energy when other metabolic pathways become less efficient with age. Along with increased CS activity, increased glycolysis might be another metabolic change that occurs as a result of muscle aging.

Future research could examine the activity of phosphofructokinase-1 (PFK-1) per the criteria examined in this study. PFK-1 is a rate-limiting enzyme in glycolysis, and understanding the effects of diel time, sex, and age on the activity of this enzyme would provide a valuable window into examining the overall metabolic changes that occur in the flight muscles of *M. sexta*. Wone et al. (2018a) suggests that glycolysis increased with age. Additional research in FBN rats and C57BL/6J mice has determined that, as beta oxidation decreases, glycolytic metabolism increases with age (Garvey et al., 2014; Houtkooper et al., 2011). Because this study examined changes in beta-oxidation and TCA activity, examining glycolysis is a next step in determining whether there is a change in the oxidative identity of aging muscle tissue.

The implications of this study could be important in understanding the circadian nature of metabolic regulation or the metabolic changes that occur during aging. Changing metabolic activity to accommodate active periods is important in both hawk moths and mammals. Metabolic diseases can make it difficult for those afflicted to maintain adequate energy throughout the day, so understanding how muscles adjust enzyme activity may be a useful step in understanding the mechanism behind metabolic syndromes. CS dysregulation may be a key component of the muscle aging process; therefore, the results of this study could provide valuable insight into reducing the impact of sarcopenia. With an aging population, curbing the effects of muscle senescence is important in developing therapies to treat the inevitable loss of muscle function due to age.

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