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DECREASED ACTIVITY OF PHOSPHOFRUCTOKINASE-1 IN FLIGHT MUSCLE CELLS OF HAWK MOTH MANDUCA SEXTA WITH AGE

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FLIGHT MUSCLE CELLS OF HAWK MOTH

MANDUCA SEXTA WITH AGE

by Owen Alvine

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ABSTRACT

DECREASED ACTIVITY OF PHOSPHOFRUCTOKINASE-1 IN FLIGHT

MUSCLE CELLS OF HAWK MOTH *MANDUCA SEXTA* WITH AGE

Owen Alvine

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The phosphofructokinase-1 (PFK-1) enzyme is important for the catalyzation and regulation of glycolysis, especially in muscle. Investigating age-related changes in PFK-1 activity will provide insights into the metabolism shifts in muscle cells of our muscle aging model. The hawk moth, *Manduca sexta,* was chosen as the model organism because of its unique endothermic, synchronous flight muscles that are more analogous to vertebrates than invertebrate species. We hypothesized that PFK-1 activity will increase in muscle cells of aged moths due to dysregulation of the mitochondria. This was predicted to change the method of energy production, by hindering oxidative phosphorylation, making glycolysis more necessary. Our results show that there is a decrease in PFK-1 activity in both sexes of hawk moth, and thus a decrease in glycolytic activity, in aged moth muscle. There was also a significant difference in PFK-1 activity between the sexes. Additionally, it was found that there was a nonsignificant difference between day and night samples. This suggests that our hypothesis was incorrect, and the activity of PFK-1 decreases as the muscle cells age ($p < 0.001$). The findings help create a better understanding of metabolism in aging.

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Introduction

Aging is a natural part of life that affects the physical body of any organism in a

multitude of ways. An interesting example of this is the calcification of the pineal gland.

With aging, the tissue of the pineal gland becomes increasingly dense with calcium which

inhibits the production of melatonin. This has been linked to insomnia and even

Alzheimer's in patients (Song 2019). Age can affect the immune system as well.

Decreased bone marrow production of B and T cells and diminished function of mature

lymphocytes in secondary lymphoid tissues cause the elderly to have much more

vulnerable immune systems (Montecino-Rodriguez et al.. 2013). Additionally, respiratory

strength decreases significantly with advanced age (Sharma 2006) causing labored

breathing and overall decrease in stamina. There is a general pattern in vertebrate skeletal

muscle where function declines as the organism ages, which can have a significant

influence on the overall quality of life. The conditions of biological aging are

characterized by a decreased ability of an organism to withstand physical challenges and

homeostatic perturbations (Fried et al. 2004). Changes occur at the cellular level as well.

For example, proteins created by muscle cells tend to misfold more often with advanced

age (Peterson et al. 2012). Cell damage (Seo 2016) and decrease in numbers of

mitochondria (Wone et al. 2018b), are seen that also affect the muscle.

Weakening muscles are not as outwardly apparent or as sudden as other

degenerative or age-related diseases such as heart disease or Alzheimer's disease, but a

weakened muscle state can increase the chance of adverse and potentially fatal health-

related states (Childs et al. 2015). Weakened muscles have been shown to contribute to osteoporosis and more falls, which greatly increases the chance of fractures in the elderly population (De Monico et al. 2011). Additionally, senescence, which means deteriorating with age, impairs cell replication making healing from wounds and fighting off infections more difficult (Campisi 2013). Muscle strength is strongly associated with increased mortality in the elderly (Mettler et al. 2002). However, loss of muscle strength and mass begins relatively early in life. Humans lose 3-8% of their muscle mass per decade after age 30 (Volvo et al. 2004). Therefore, it important to study and understand the mechanisms underlying age-related decrease in muscle function. Advancements in this area could produce future treatments and therapies to improve quality of life and even extend the health span.

This aging process of skeletal muscle cells has many etiologies, and narrowing it down to a single culprit is not possible. The disruption of metabolic processes seems to have a large impact. Ortega et al. (2015) has shown that the metabolic demand for middle aged people during moderate exercise is much less than the metabolic demand for older people doing the same activities. This suggests a sharp decrease in metabolic efficiency in the elderly compared to the middle aged. Age-related changes in mitochondrial function may, in part, account for the difference in metabolic demand. Decreased mitochondrial function is shown to be linked with an increase in reactive oxygen species (ROS), which can contribute to a decrease in muscle function (Short et al.., 2005).

To study the metabolism in muscle, it is important to find a suitable model organism. Testing physiology too different from our own will render the findings meaningless when applying the results to human aging. Mammals are the preferred model organisms. However, the time, money and resources required to raise and house most mammalian species to senescence are major disadvantages in aging research. Insects are suitable subjects for many types of research, while being easy to access and less time consuming to acquire and raise (Wone et al. 2018b). Their short lifespan also makes them ideal for studying processes of aging, because it takes less time for the specimens to reach maturity. The negatives of using insects are that their biology is vastly different from mammals, so findings do not always translate well for application to other animals or humans (McMahon 2017). However, Wone et al.. (2018a) reported that one species of moth represents a good model to study age-related skeletal muscle function. Although not generally seen as similar to humans, the unique endothermic flight muscles of the hawk moth, *Manduca sexta*, provide a good model for vertebrate muscles (George et al. 2011). These muscles are synchronous and contract with each stimulus of a neuron, like our own. This is unlike other insects which simply require an initial pulse to generate continuous contractions (Josephson et al. 2000). Taken together, these findings suggests that *Manduca sexta* provides an ideal invertebrate model to study and potentially apply the findings to human research because of the ease of accessibility and maintenance, short lifespan, and similar to vertebrate muscles.

After finding the most appropriate model to study, the muscle function of *Manduca sexta* can be analyzed throughout the aging process. PFK-1 is the rate limiting step for

glycolysis and is indicative of the glycolytic process. Glycolysis increases with increasing age, as an attempt to keep appropriate energy production, because aerobic metabolism slows (Rivera et al. 2019). Specifically, the changes in the use of the enzyme PFK-1 in glycolysis can be measured to understand how metabolism changes in the short lifespan of *Manduca sexta*

Background

Research into metabolic and muscular aging is at the forefront of medical science, with a focus on how to increase quality of life well into the elderly years. This requires an understanding of how muscle aging occurs and the accompanying physiological changes. If these are better understood, then the negative consequences of aging may be effectively mitigated. It is well-known that aging is the result of damage to DNA, leading to downstream effects for virtually all metabolism in the body. Some of the DNA damage is caused by the shortening of telomeres, which are the caps at the ends of chromosomes. Too much damage of these protective structures can lead to harm of vital genes (Aubert et al.., 2008). The rate of telomere shortening has been linked to the level of oxidative stress from ROS and dysfunction of mitochondria can accelerate telomere shortening (Stuart et al. 2006). Research conducted by Wone et al. (2018a), suggested an increase in glycolysis and TCA cycle in the flight muscles of *Manduca sexta* with aging. This was found because of a decrease in *Beta*-oxidation in flight muscle cells. This shows a change in the route of energy acquisition over time in these organisms.

Previous research by Becker and Rudolph (2021) concludes that as biological aging progresses, enzymes decrease their activity. This finding suggests that mitochondrial enzyme activities would decrease with age and thus less energy is produced. In *Apis mellifera* (honeybees)*, a more direct PFK to age relationship was established. PFK was lowest in the middle aged bees aged 0-1 day and highest in the old bees aged 19-20 days (Greeno-Shannin 1988).

With rat studies, analysis of muscle tissue has found that with advanced age, the amount of myosin heavy chains and mitochondrial proteins decrease. The lack of myosin heavy chains is consistent with our knowledge of sarcopenia, which is the gradual loss of muscle with age. The decrease activity of mitochondrial proteins supports my hypothesis and suggests that mitochondria dwindle in the later years of life (Nair 2005). This might mean that glycolysis must take an increasing role in metabolism and thus an increase in PFK-1 activity. That same study looked at the potential mechanisms for aging in muscle. It was found that the presence of insulin increased the number of mitochondrial proteins along with in increase in muscle ATP production. It was mentioned however that the effects of glucose remain to be seen.

In a study conducted by Palla et al. (2021) they explored the genetic and metabolic mechanisms behind sarcopenia. It was concluded that decreased expression of the inflammation-reducing enzyme 15-hydroxyprostaglandindehydrogenase led to a decrease in the protein synthesis and degradation ratio and therefore a decrease in muscle atrophy.

Interestingly, a human study of various skeletal muscle found that there was a change in the activity of two important glycolytic enzymes, those being hexokinase and pyruvate kinase. Hexokinase was found to decrease in the rectus abdominis, and pyruvate was seen to increase in the gluteus maximus (Pastoris, 2000). These results suggest that different muscles utilize glycolysis in different ways with age.

Wone et al.. (2018a) has suggested an increase in glycolysis and TCA cycle in the flight muscles of *Manduca sexta* with aging, which points to a changing demand in the energy needs of these organisms. Additionally, Kim et al. (2017), showed that skeletal muscle aging is strongly correlated with a loss of both metabolic efficiency and mitochondrial biogenesis. These results indicate that the aging organism cannot effectively create new mitochondria for adenosine triphosphate (ATP) production. Kalyani et al. (2014) found that metabolism shifts from more aerobic to anaerobic pathways. This is most likely due to the accumulation of ROS from the electron transport chain. ROS are very volatile molecules that are produced from aerobic metabolism. Surprisingly, 1-5% of all oxygen consumed during aerobic respiration is reconstructed into a superoxide due to leakage from the electron transport chain (Kong et al. 2014). An ROS, also known as a free radical, is a superoxide, with one or more unpaired electrons in its valence shell. ROS are reduced by other molecules for stability. This reaction creates a free radical out of the donor molecule which causes a self-sustaining chain reaction that can ultimately damage the cell. This buildup of ROS is a hallmark of aging in organisms, because the energy production is deteriorating (Stefantos 2017).

Further burdening cells, ROS can cause macromolecule damage and potentially lead to cancer and other ailments (Ray et al. 2012). They can interact with proteins or lipids directly and affect the molecule's ability to carry out cellular processes. Genetic studies in mice indicate that individuals with mutations in genes designed to regulate ROS, leading to increased concentrations, have rapid decline in stem cell renewal. Conversely, mice lacking in genes that increase the ROS have extended life spans (Kong et al. 2014).

Slow deterioration of mitochondria during aging indicates that the organism will slowly lose the ability to use aerobic metabolism by way of the Krebs cycle and electron transport chain (Boengler et al. 2017). Because the electron transport chain and the Krebs cycle allow for 34 ATP per glucose molecule, it is the most efficient way for a cell to produce energy. The alternative to this process is by way of the glucose-cleaving chemical reaction of glycolysis to produce 2 ATPs per glucose. Glycolysis also produces pyruvate as the substrate in the Krebs cycle, but without the mitochondria, its potential is not accessed as the citric acid cycle takes place within the mitochondria.

PFK-1 is an important enzyme in glycolysis. It is the third enzyme in the reaction and is responsible for turning fructose-6-phosphate into frustose-1/6-bisphophate. This step is known as a committed step because it requires ATP to proceed and does not exist within a state of equilibrium like some other steps of glycolysis (Jenkins et al. 2011). Thus, the measure of PFK-1 activity is indicative of the rate of glycolysis as the reaction proceeds linearly with the concentration of PFK-1. Measuring the amount of PFK-1

activity directly correlates with the reaction rate of glycolysis. Measuring the PFK-1 activity in moth muscle tissues might provide an index of glycolytic contributions to and age-related shifts in energy production. Mammalian studies on the enzymatic processes in sheep have found that the levels of PFK-1 greatly increased with age (Gardner et al.., 2007).

Methodology

This study utilized both male and female adult moths, at different ages in their life cycle, specifically middle aged and advanced age. The effects of circadian rhythms on metabolic functions were addressed as samples were obtained during the day and night. This was done because the metabolic processes are associated with activity, sex, and age and it has been established that sex is an important factor in the lifespan of these moths (Wone et al. 2018a). Twenty-three hawk moth samples were collected. 10 were male and 12 were female, 11 each were middle aged and aged, and finally 10 were collected during the night and 12 were collected during the day. For females, age 4 and 7 days were studied at both the day and night periods of the light cycle. For males, age 2 and 5 days were studied at both the day and night. Three biological replicates were gathered for 3 of the categories (male old, female middle aged, and female old). For the middle aged males, only 2 biological replicates were obtained due to a lack of suitable sample tissue. At the respective ages, moths were euthanized by decapitation and the muscles were rapidly frozen in liquid nitrogen to preserve muscle enzymes. The muscles were then ground into a fine power using a mortar and pestle with the aid of liquid nitrogen to keep the enzymes frozen.

Enzyme Spectrophotometric Assay

The activity of PFK-1 was investigated in this study as it is the enzyme converting fructose-6-phosphate into fructose-1,6-bisphosphate. The enzyme PFK-1 is the committed step because it is the most regulated step of glycolysis and thus the most likely step to be hindered if glycolysis was halted. The production of fructose-1,6-bisphosphate, from PFK-1 activity can be measured by determining the loss of nicotinamide adenine dinucleotide (NADH) using a spectrophotometer set at 340nm.

ADP + Substrate ---(Enzyme mix)-→ AMP + NADH → Light (λ = 340nm)

As the rate of NADH lost is measured, the amount of PFK-1 that was active in the muscle during its time in the assay reader can be calculated. The reaction in question is the catalyzation of ADP and substrate to AMP and NADH. The substrate with the PFK-1 accepts a phosphate from the ADP molecule creating both AMP and NADH as products.

The flight muscles were then assayed on a Eppendorf Microplate UV-VIS, 96/F plate (Eppendorf, Hauppauge, NY). The plate consisted of separate sections split up between age, diel time and sex. 10 mg of flight muscle sample was homogenized with 100 µl of ice cold homogenizing buffer. After centrifuging, the supernatant was collected and 2 µl substrate was combined in each well with 42 µl assay buffer, 2 µl PFK enzyme mix, 2 µl PFK developer, and 2 µl ATP. Absorbance was read on kinetic loop at 39˚C for 40 minutes with an Accuskan GO UV/Vis Microplate Spectrophotometer. Fisherbrand model #14-377-579 (Thermo Fischer Scientific, Pittsburg, PA).

Once the data were collected, they were transferred to an excel spreadsheet. A portion of the slope was chosen for each to represent the rate of absorbance used by the

muscle sample. The equation B/(ΔT x V) x dilution factor was used to calculate the PFK-1 Activity. B is the NADH amount from the standard curve. It was gathered by measuring the absorbance of known NADH concentrations and creating a slope from the data. The slope of this graph was calculated to be y = 0.0305X-0.0052. Rearranging the equation for X to find the concentration from the absorbance, the equation becomes $X = (y+0.0052)/0.0305$. ΔT is the reaction time of the chosen slope (5.84 minutes), and V is the sample volume (0.002 ml). This data was then applied to a three-way ANOVA test with age, sex and diel time as the variables.
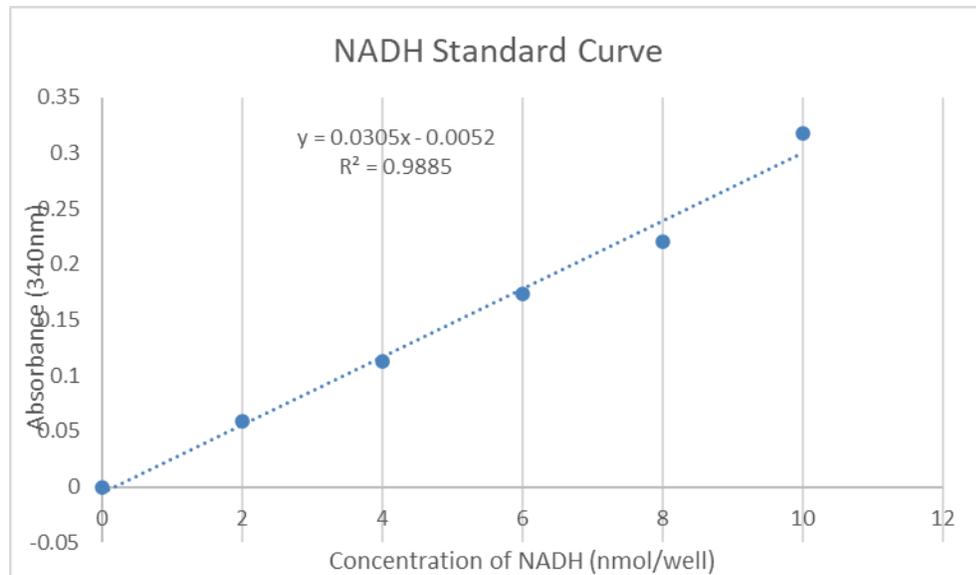

Figure 1: NADH standard curve with slope equation presented. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate per minute at pH 8.2 at 39° C.

ANOVA				Alpha	0.05	
	SS	df	MS	F	p-value	p eta-sq
A	66.98268	1	66.98268	1.223395	0.28734	0.080363
B	543.421	1	543.421	9.925229	0.007086	0.414844
C	6311.913	1	6311.913	115.283	3.84E-08	0.89171
A x B	87.21988	1	87.21988	1.593014	0.227518	0.102162
A x C	609.7747	1	609.7747	11.13714	0.004887	0.443055
B x C	1622.322	1	1622.322	29.63065	8.66E-05	0.679125
A x B x C	-627.001	1	-627.001	-11.4518	0.00452	-4.49398
Within	766.5208	14	54.75148			
Total	9381.153	21	446.7216			

Table 1: Results of ANOVA statistical analysis of data. A = sex, B = diel time, C = age.

Results

An old male nighttime sample was omitted for having uncharacteristic absorbance values and one technical replicate was removed for 3 other samples. The remaining 2 technical replicates were suitable. With the three-way ANOVA results complete, it was apparent that both age and diel time had a significant difference in PFK-1 activity (p =0.00000003, F = 115.3 and p=0.007, F = 9.9 respectively: figure 8/9 and 7). No statistical differences were found between moths of different sexes (p >0.05: figure 6). However, there was a three way significant interaction between all the categories (p = 0.0045)

The average PFK-1 activity can also allow some insight into the difference in metabolism between the 8 different groups. The averages for the male day middle age moths were 133.5 U/ml and male day old moths average was 104.19 U/ml (P= 0.0769, figure 3). For the male night middle aged, the average was 131.1 U/ml and 88.05 U/ml for the old group (P = 0.085, figure 4). The female night middle aged moths had an average of 136.7 U/ml and the female night old moths average was 89.8 U/ml (P = 0.041, figure 5). Finally, the female day middle aged moths had an average of 133.4 U/ml and the female day old moths had an average of 114.01 U/ml (P = 0.277, figure 2). Additionally, the average for the middle aged moth group was 13389.85 U/ml and the average for the old moths group was 100.02 U/ml.

Additionally, females separated by age and diel time show a pattern of both middle aged and day moths having a significant increase over the counterparts (figure 10).
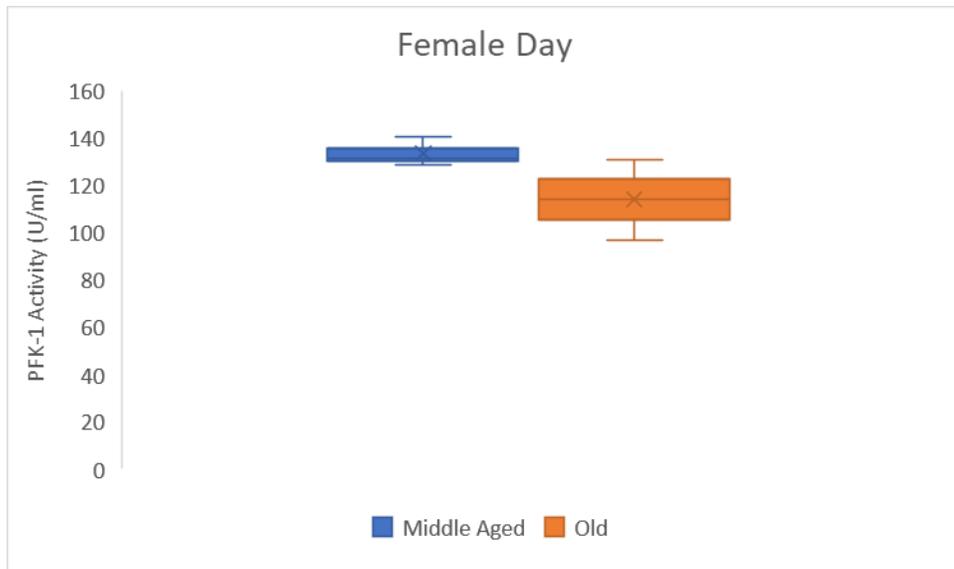
Figure 2: Female day middle aged moths (n=3) were compared to the female day old moths (n=3). P=0.277. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate per minute at pH 8.2 at 39° C.
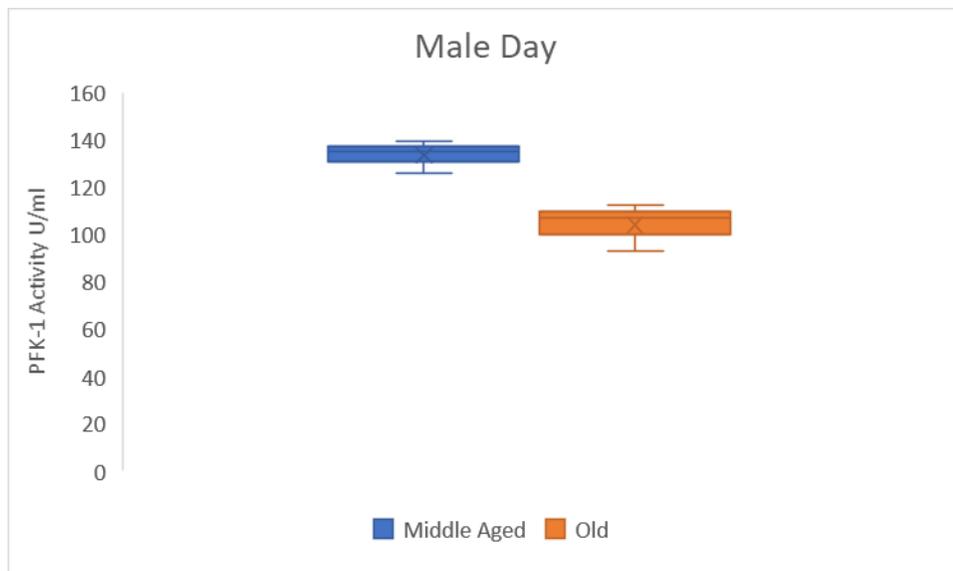

Figure 3: Male day middle aged moths (n=3) were compared to the male day old moths (n=3). P=0.0769. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate per minute at pH 8.2 at 39° C.
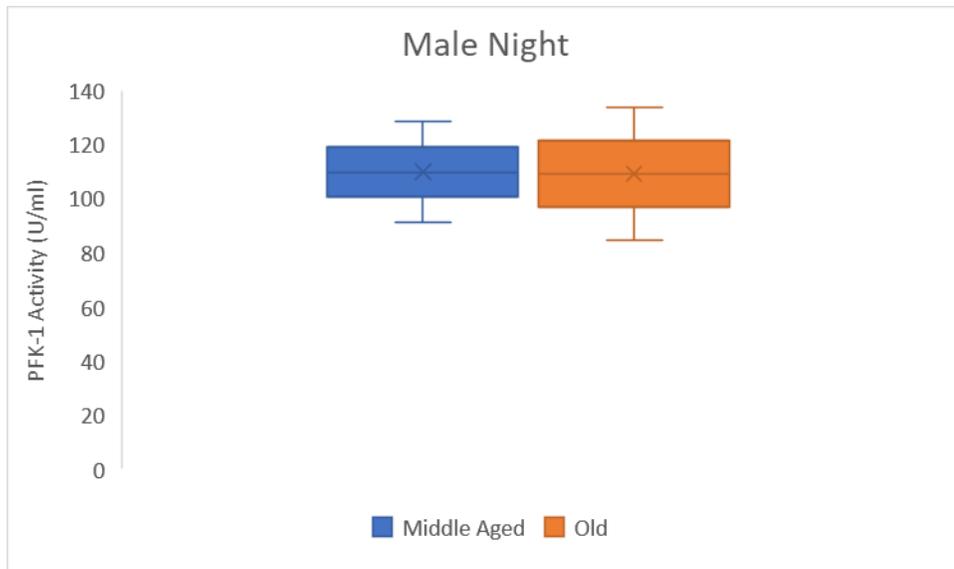
Figure 4: Male night middle aged moths (n=2) were compared to the male night old moths (n=2). P=0.085. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate per minute at pH 8.2 at 39° C.
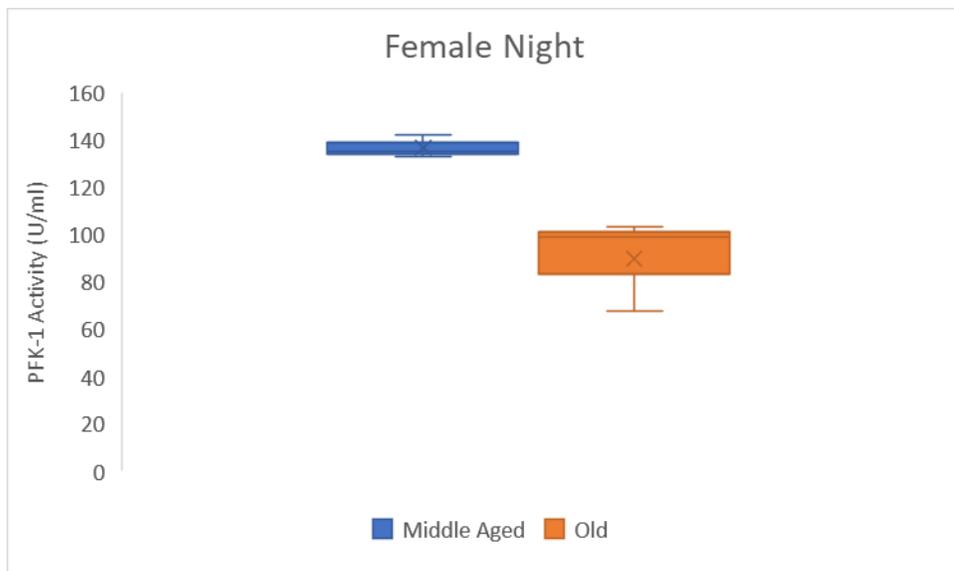

Figure 5: Female night middle aged moths (n=3) were compared to the female night old moths (n=3). P= 0.041. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate minute at pH 8.2 at 39° C.
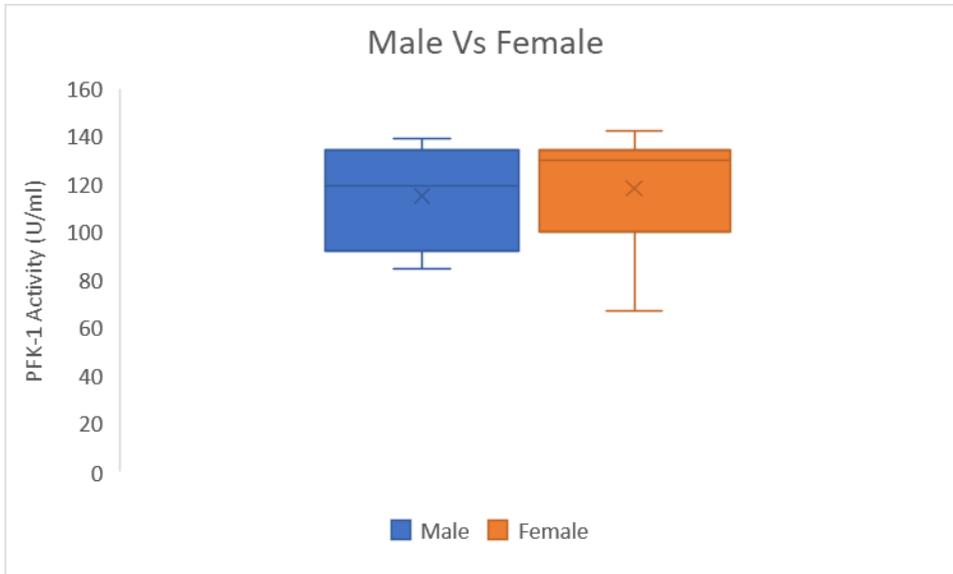
Figure 6: Male moths (n=10) were compared to the female moths (n=12). P=0.49. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate minute at pH 8.2 at 39° C.
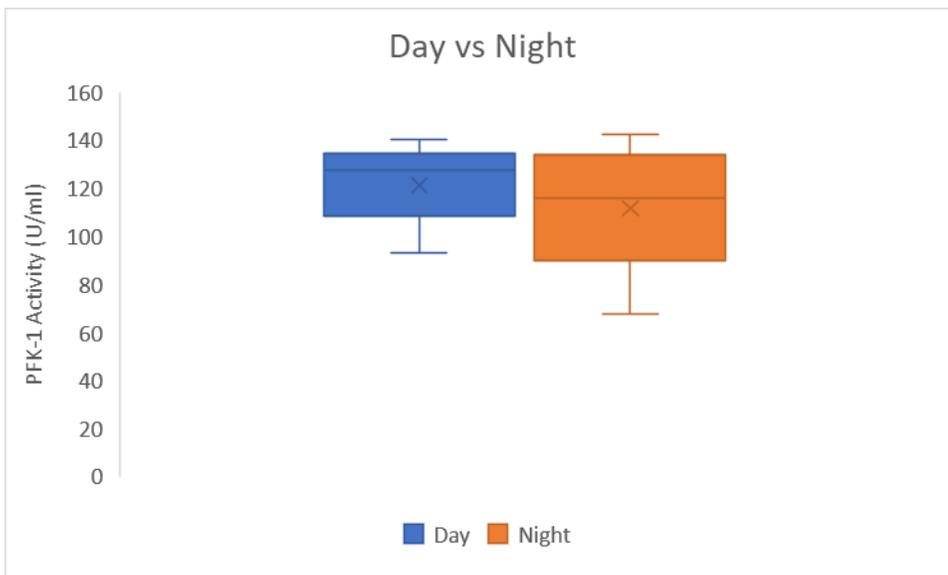

Figure 7: Day samples (n=12) were compared to the night samples (n=10). P=0.007. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate minute at pH 8.2 at 39° C.
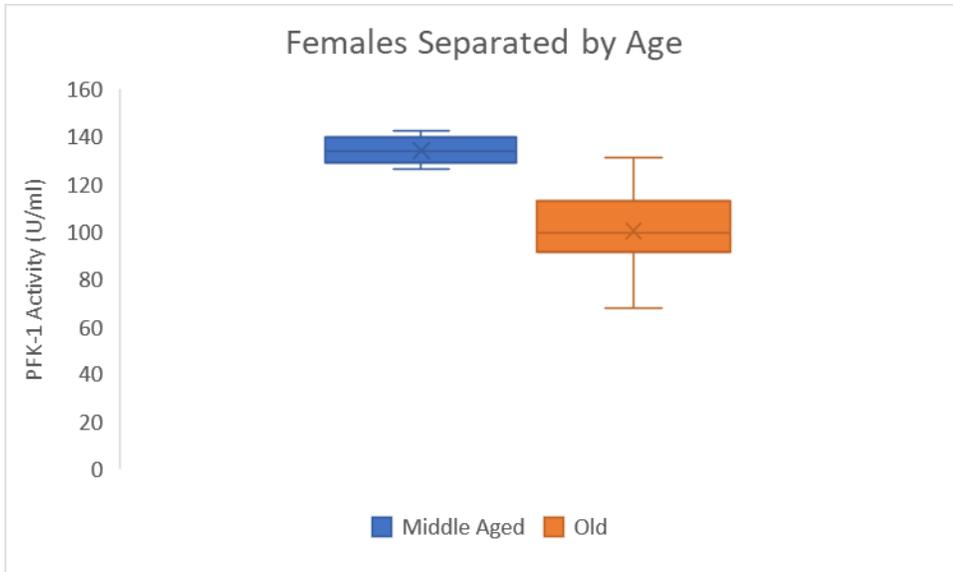
Figure 8: Females separated by age (n=22). P=0.00013. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate minute at pH 8.2 at 39° C.

Figure 9: Males separated by age (n=10). P=0.0046. Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate minute at pH 8.2 at 39° C.
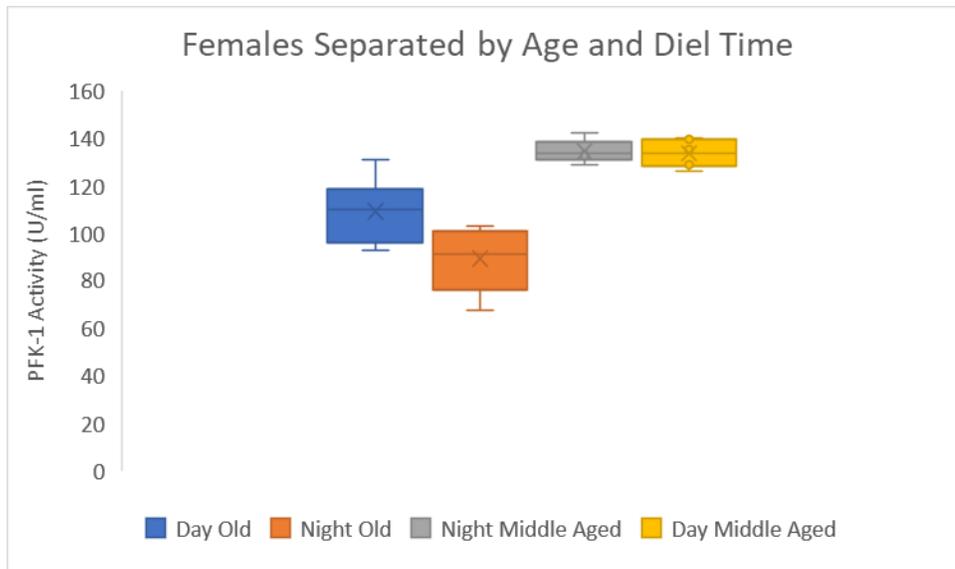
Figure 10: Females separated by age and diel time (n=23). Quartiles are calculated with internal medians. Upper and lower whiskers represent the maximum and minimum distributions of the data. Unit (U) definition - One unit will convert 1.0 μmole of fructose-6-phosphate to fructose-1/6-bisphosphate minute at pH 8.2 at 39° C.

Discussion

In this study, the PFK-1 enzyme activity was measured in moth muscle at two different ages, middle aged and advanced age, and looked at differences in diel time and sex. The results are inconsistent with the previous hypothesis of this study and show PFK-1 activity is lower in in advanced age moth muscle compared to middle age. It was also found that differences in diel time have an impact on the PFK-1 activity with the day samples showing higher activity than the night. The differences in sexes were nonsignificant suggesting that males and females use roughly the same amount of glucose. This decrease in PFK-1 activity might be indicating an overall decrease in enzyme function as moths age, possibly due to decreased expression and increased oxidation (Becker and Rudolph 2021).

These results are somewhat consistent with McMahon (2017). It was found that citrate synthase in females, an enzyme that converts oxaloacetate and pyruvate into citrate for the TCA cycle, increased with the age of the moth. It would suggest that as the cell moves towards more aerobic methods of metabolism, it would shift away from anaerobic methods (glycolysis).

Another potential explanation for the results is that as the flight muscles of the moth age, they atrophy. This atrophy could result in simply less real estate for glycolysis to take place. If that were the case, it would be expected that PFK-1 activity would be lower. Wone et al. (2018b) found that the hawk moths muscle mass decreases with age regardless of whether they were fed. Future research could study the cytoplasm of middle aged and aged hawk moth muscle and compare this to the amount of PFK-1 activity in those muscles as they age.

Mitochondria fuse with age, leading to fewer mitochondria, and decreased oxidative phosphorylation (Wone et al. 2018b). The results of this study seem to indicate that glycolysis does not increase to compensate for the reduction in aerobic metabolism. Past research by Wone et al. (2018a) showed that aged female moths had a 3.09-fold increase in TCA cycle metabolites. This suggests that the tricarboxylic acid cycle has a larger metabolic need than other forms of metabolism, such as glycolysis.

Conclusion

The results of these experiments provided some insightful information about the activity of glycolysis in muscle as it ages. It was determined that a significant decrease in PFK-1 activity is correlated with aging flight muscles in the moth species *Manduca sexta.* In the future, more knowledge about muscle aging will be useful in attenuating and treating the effects of advanced age. As discussed, changes to muscles throughout life can be a very detrimental effect of aging. Metabolic diseases can make it difficult to maintain a healthy homeostasis. Improved understanding of how aging muscles handle mitochondrial changes will be useful in mitigating and managing some of the negative consequences of growing old.

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