THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

SEX DIFFERENCES IN AUTOPHAGIC PROTEIN EXPRESSION

GABRIELLE AGUILAR
SPRING 2020

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biology
with honors in Letters, Arts, and Sciences

Reviewed and approved* by the following:

Steven Bloomer
Associate Professor of Biology
Thesis Supervisor

Eric Ingersoll
Associate Professor of Biology
Faculty Reader

David Ruth
Associate Professor of History
Honors Advisor

* Electronic approvals are on file.

ABSTRACT

In general, the average female lifespan is longer than the average male lifespan. These lifespan differences are supported by the differences in oxidative stress and inflammation between the two sexes. The aim of this study was to observe autophagic sex differences and provide a mechanism that would suggest an explanation for the sex differences in oxidative stress and inflammation. Autophagy can affect inflammation through removal of inflammasome components from the cell, and oxidative stress through degradation of defective mitochondria, thus reducing peroxide production. One of the vital autophagy proteins is sequestosome 1 (also known as SQSTM1 or p62), which is an autophagy substrate whose levels can indicate the extent of autophagy within the cell. The level of this protein is inversely proportional to autophagic activity. This study utilized the livers of male and female mice at 25-weeks of age. As was expected, females showed a lower amount of p62 protein, suggesting that autophagic flux is greater in females.

TABLE OF CONTENTS

ABBREVIATIONS iii

LIST OF FIGURES v

ACKNOWLEDGEMENTS vi

Chapter 1 Introduction 1

Chapter 2 Review of Literature 3

The female sex 3
Sex differences in inflammation in the liver 4
Sex differences in oxidative stress in the liver 4
Autophagy 6
Types of autophagy 7
Macroautophagy 7
Chaperone-mediated autophagy 7
Organelles involved in autophagy 8
Proteins involved in autophagy 9
P62.. 9
LC3 9
Role of macroautophagy in preventing oxidative stress 10
Degradation of dysfunctional mitochondria 11
Role of macroautophagy in preventing inflammation 11
Autophagy in female animals 12
Autophagy in male animals 14
How sex differences in autophagy might explain why females develop less oxidative stress and inflammation 15

Chapter 3 Materials and Methods 15

Sample Preparation and Immunoblotting 15
Band Determination 16
Statistics 17

Chapter 4 Results 18

Protein Quantification 18

Chapter 5 Discussion 23

Chapter 6 Future Directions 26

BIBLIOGRAPHY 28

LIST OF ABBREVIATIONS

1° Ab	Primary antibody
2° Ab	Secondary antibody
AFB_1	Aflatoxin B1
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
ATG	Autophagy-related
Az-PC	Azelaoyl phosphatidylcholine
CCSMC	Corpus cavernosum smooth muscle cells
FTLD	Frontotemporal lobar degeneration
H_2O_2	Hydrogen peroxide
Hsp40	Heat-shock protein 40
IL	Interleukin
kDa	kiloDalton
LAMP2A	Lysosomal-associated membrane protein 2A
LC3	Microtubule-associated protein 1A/1B-light chain 3
MEF	Mouse embryonic fibroblasts
MIF	Macrophage migratory inhibitory factor
NAFLD	Non-alcoholic fatty liver disease
NRF2	Nuclear factor erythroid 2-related factor 2
OVX	Ovariectomy

p62	Sequestosome 1
PAF	Platelet-activating factor
PE	Phosphatidylethanolamine
ROS	Reactive oxygen species
SEM	Standard error of the mean
SQSTM1	Sequestosome 1
TBST	Tris-buffered saline
TNF	Tumor necrosis factor

LIST OF FIGURES

Figure 1: Western blots for p62. The first lane shows the positive control ("+") for p62 from the neuroblastoma cell line. The second lane ("Sample") shows a liver sample.................. 19

Figure 2: Western blot for LC3-II/LC3-I protein. The first lane shows the positive control ("+") for LC3-II/LC3-I from the neuroblastoma cell line. The second lane ("Sample") shows a liver sample.. 20

Figure 3: Representative Western blots for the p62 protein in the male and female mice (top panel). Quantitation of p62 protein abundance in males and females (bottom panel). Results are presented as the ratio of p62 brightness to the density of the Ponceau stain. Results were further normalized to the male group, which was given a value of 1. Data are expressed as means + SEM.. 21

Figure 4: LC3-II/LC3-I protein ratio abundance in males and females. Results are presented as the ratio of p62 brightness to the density of the Ponceau stain. Results were further normalized to the male group, which was given a value of 1. Data are expressed as means + SEM. 22

ACKNOWLEDGEMENTS

I would like to thank Dr. Steven Bloomer for allowing me to join his research and for his support and patience throughout the entire thesis process. Secondly, I would like to thank Dr. David Ruth for his advice throughout my honors career at Abington. Thirdly, I would like to thank Dr. Eric Ingersoll for his time and guidance in my thesis work.

Finally, I would like to thank my family and friends for their endless encouragement throughout the journey towards my undergraduate degree.

Chapter 1

Introduction

It has been established that, in general, the average female lifespan is longer than the average male lifespan (1). This difference in lifespan is thought to be due, in part, to lower levels of inflammation and oxidative stress in women compared to men. The inflammatory response and oxidative stress are linked within the body; oxidative damage will result in intracellular damage and inflammation as a result of the production of free radicals or reactive oxygen species (ROS). Excessive amounts of oxidative stress and inflammation can cause damage to macromolecules, and if not cleared, these damaged macromolecules will cause cell death. One of the intracellular mechanisms to clear these macromolecules that have lost their function is autophagy; autophagy will degrade damaged molecules and help maintain the survival of the cell in both normal and stressful conditions, and its resulting products will be used to synthesize new organelles. Autophagy also prevents excessive inflammation and oxidative stress (17, 21). Potential sex differences in autophagy could influence the susceptibility to inflammation and oxidative stress. Some studies have investigated sex differences in autophagy in the cardiovascular and nervous systems, but few have focused on the liver, which is a crucial organ for overall homeostasis.

One of the vital autophagy proteins is sequestosome 1 (also known as SQSTM1 or p62), which is an autophagy substrate whose levels can reflect the extent of autophagy within the cell (9). The level of this protein is considered to be inversely proportional to autophagic activity, since it is degraded in autophagosomes (9). Another vital autophagy indicator involves the ratio

between the lipidated microtubule associated proteins 1 and 2. The cytosolic form of the lipidated microtubule-associated protein 2 light chain 3 alpha (or LC3-II) is the most commonly used protein marker for detecting autophagy; LC3-I will conjugate with phosphatidylethanolamine (PE) to form LC3-II, which is then recruited to the membrane of autophagosomes where p62 will link cellular cargo onto it. It is accepted that the larger the LC3-II/LC3-I ratio is, the greater the amount of autophagosome production and thus autophagic flux. However, measuring the amount of LC3-II protein in the cell can be difficult, since LC3-II on the outer membrane of the autophagosome is cleaved off by the Atg4 gene while the LC3-II protein on the inner membrane is degraded by the lysosomal enzymes, as observed by Mizushima *et. al* (26). This degradation can result in a low LC3 content in the autolysosome and thus, the same amount of LC3 would be detected even if there were differences in the rate of the autophagic process since LC3-II is facilitating the process on the cytoplasmic side and not being consumed by autophagy (26).

The goal of this study was to determine potential sex differences in autophagy by measuring the levels of p62 and LC3 proteins within the livers of 25-week old male and female SAMP8A strain mice. Differences in the expression of these proteins might demonstrate differences in autophagy, which could suggest an explanation for the sex differences in oxidative stress and inflammation.

Chapter 2

Review of Literature

The female sex

In general, the average female lifespan is longer than the average male lifespan (1). Later in life, men will have a greater chance of developing cardiovascular diseases, high blood pressure, and Parkinson's disease (2). The similarities between many of these diseases lie within their etiologies; oxidative stress and inflammation will play a large role in the development of these diseases within the two genders as they age (3, 25). Autophagy is a conserved cellular eukaryotic process occurring in the lysosomes of cells; the function of lysosomes is to degrade the intracellular macromolecules and organelles that have lost function as a result of stressors, such as inflammation and oxidative stress. As a result, autophagy will decrease inflammation and oxidative stress. Potential sex differences in autophagy could influence the susceptibility to inflammation and oxidative stress. Additionally, sex differences could influence autophagic flux in multiple organs, including the liver. The purpose of this study was to observe the difference in autophagy between female and male mice with focus on the hepatic response, and determine if these differences in autophagy could explain the sex differences in the inflammatory and oxidative stress responses.

Sex differences in inflammation in the liver

The inflammatory response, as a result of injury or invasion by foreign substances, can result in the excessive production of pro-inflammatory, cytotoxic cytokines and molecules. Autophagy will counter-act inflammation to maintain cellular homeostasis, resulting in a decrease in cytokine production and a decrease in cell death by cytokines (4). As noted by Bloomer *et. al*, there is a sex difference in the basal expression of hepatic inflammatory proteins in the liver; female mice displayed lower levels of ICAM-1, MCP-1, and COX-2 (5). They showed that there are prevalent sex differences in the susceptibility to metabolic diseases, including non-alcoholic fatty liver disease (NAFLD), which is associated with inflammation (27). The disease is sexually dimorphic and is generally seen at a higher prevalence in males. This study suggests that the susceptibility of female mice to inflammation and liver disease is lower than that of male mice (5).

Sex differences in oxidative stress in the liver

Oxidative stress is a condition of excessive production of free radical molecules, which are molecules with unpaired electrons. Mitochondria will produce free radicals normally, but if mitochondria are dysfunctional, they will produce more, leading to intracellular damage. Free radicals can react with macromolecules in the cell such as lipids, proteins, and nucleic acids, leading to oxidative damage of these molecules. One particular example of oxidative damage is the oxidized phospholipid, azelaoyl phosphatidylcholine (Az-PC), which is produced by liver cells when low density lipoprotein particles are oxidized. Therefore, it can be used as a marker of

oxidative stress. Furthermore, Az-PC can cause depolarization of mitochondria, which can augment cellular damage (6).

Oxidatively modified phospholipids like Az-PC are increased in the circulation in mild oxidative stressed with aging, and in males in comparison to female mice (6). Degradation of these phospholipids by rapid transport into the liver is unchanged between sexes, so circulating levels measured in this experiment reflects the continuously increased production of these phospholipids and movement into the circulatory system (6). As a result of an increase of oxidative stress, the rate of efflux of Az-PC and platelet-activating factor (PAF) from the liver were increased in the circulation (6). With this being said, the researchers observed that the production rates of both Az-PC and PAF increased at the same rate and concluded that the rate of Az-PC and PAF production (that is, oxidation and inflammation) are both enhanced in older, male animals. This result, along with the increase in PAF production, suggests that the male sex promotes on-going oxidative stress in the liver that is greater in males than females (6).

Estrogen therapy has been found to have a protective effect against the production of free radicals by mitochondria. As observed by Borras *et. al*, peroxide production, which is an example of a free radical, is higher in liver mitochondria from male rats than female rats, and this result was even more pronounced after sampling the brains of the male rats (7). In order to determine the effect of estrogen on peroxide production by mitochondria, the researchers observed the oxidant production of ovariectomized female mice, which are mice lacking ovaries and thus, estrogen. The rate of production of peroxides by mitochondria from these ovariectomized female rats was similar to the production of male mice (7); after estrogen replacement therapy, the increase peroxide production due to the ovariectomy was completely prevented (7).

Both inflammation and oxidative stress are stressors to the cell. In order for the cell to maintain survival and homeostasis through these stressful conditions, the cell will undergo the process of autophagy in order to protect the cell and prevent these stressful conditions from occurring (4).

Autophagy

Autophagy is a conserved cellular eukaryotic process occurring in lysosomes; the function of lysosomes is to degrade the intracellular macromolecules and organelles that have lost function. The resulting products of autophagy are used to synthesize new organelles, which helps to maintain cellular homeostasis. A family of genes within the cell known as AuTophaGy (ATG) genes, will regulate the formation of the autophagosome, which is the specialized double-membrane vesicle responsible for the delivery of defective cytoplasmic components to the lysosome. These cytoplasmic components can include damaged organelles, invading microorganisms, and abnormal intracellular proteins, and will be identified by receptor proteins within the cell to form the double membrane in order to sequester the material from the cytosol. Autophagosomes will then use a microtubule track to travel to and fuse with lysosomes, where the luminal hydrolases will degrade the cargo (4).

Types of autophagy

Macroautophagy

There are three main mechanisms in which autophagic material can be broken down and delivered to lysosomes. Macro-autophagy, herein known as autophagy, is the main form of autophagy that allows for the degradation of proteins, protein aggregates, lipids, carbohydrates, and damaged organelles. These materials are then fused with lysosomes to be degraded by hydrolases, and can eventually become recycled to sustain cellular survival. This type of autophagy is more selective than micro-autophagy, but less selective than chaperone-mediated autophagy (8).

Microautophagy

Micro-autophagy, otherwise known as endosomal microautophagy, is a less-selective and lesser known form of autophagy that involves the direct invagination of the lysosomal membrane around the cytosolic proteins/material that is to be degraded into late endosomes. The material will then undergo degradation in this compartment or in the lysosomes upon endosome and lysosome fusion (8).

Chaperone-mediated autophagy

Chaperone-mediated autophagy is the most selective form of autophagy and involves the degradation of soluble cytosolic proteins that contain the common pentapeptide KFERQ or KFERQ motif analogue amino-acid sequences. Under stresses such as oxidative

stress, the chaperone protein Hsc70 will bind to different co-chaperones such as Hsp40 (which is a heat-shock protein of 40 kiloDaltons) within the cytosol (8). This binding will form a macromolecular complex consisting of Hsc70, its co-chaperones, and the protein substrate that promotes the ability of the heat shock protein to bind to the KFERQ motif analogue in substrate cytosolic proteins. Once this motif is recognized, it will become co-routed with the macromolecular complex, and co-chaperones to the cytosolic domain of the lysosomal-associated membrane protein 2A (LAMP2A), which is the specific receptor for this type of autophagy (8). This binding will induce LAMP2A to redirect from its original position in the lipid rafts to a more fluid membrane area, where it will dimerize then trimerize to form a channel allowing for the transport of the protein substrate into the lumen of the lysosome (8). Once these proteins are transported to the lysosomal lumen, the material is degraded by lysosomal proteases, and the products of this degradation (amino acids) are recycled in order to maintain cellular homeostasis and/or promote survival (8).

Organelles involved in autophagy

Macroautophagy involves the formation of double vesicle membraned autophagosomes that will surround a portion of the cytosol to be degraded. This portion of cytosol includes organelles to be degraded, such as the mitochondria. Autophagosomes, or the double membrane vesicles that form around autophagic cargo, will fuse with lysosomes in order for degradation to occur. (8).

Proteins involved in autophagy

P62

Sequestome 1 (also known as SQSTM1/p62), is an autophagy substrate whose levels can measure the extent of autophagy within a cell. Since SQSTM1 is degraded in autophagosomes, the level of this protein is considered to be inversely proportional to autophagic activity (9). However, the expression level of p62 can fluctuate depending on transcriptional and post-translational factors, so it is advised by numerous studies that more than one marker for autophagy be used when researching the process (10). For example, in response to the oxidative stressor hydrogen peroxide (H_2O_2), the transcription factor responsible for p62 regulation, NRF2, will bind to the antioxidant-responsive element located on the promotor of p62, leading to an increase in the abundance of p62 mRNA. This response juxtaposes the relationship of autophagy and p62; given that p62 is degraded during autophagy, one would expect that an increase in oxidative stress would decrease the amount of p62 and thus increase the level of the autophagic response (11).

LC3

LC3 is the most commonly used protein marker for detecting autophagy. The cytosolic form of LC3, known as LC3-I, conjugates with phosphatidylethanolamine (PE) to form LC3-II, which is recruited to the membrane of autophagosomes. SQSTM1/p62 will link cellular cargo to LC3-II. It is accepted that the amount of LC3-II is proportional to the extent of autophagosome

formation and thus the extent of autophagy. This conversion of LC3-I to LC3-II can be detected by Western blot analysis (9).

As a result of fusion of the autophagosomes with lysosomes, subsequent degradation of the luminal contents occurs, which is known as autophagic flux. This differs from autophagosomes biogenesis, or the formation of the autophagosome. Since autophagosome biogenesis recruits LC3-II into the membrane, levels of LC3-II can be measured as a result of autophagosome formation; increasing autophagosome formation increases the amount of LC3-II. On the contrary, increasing autophagic flux, and thus the fusion of autophagosomes with lysosomes, decreases the amount of LC3-II (9).

Role of macroautophagy in preventing oxidative stress

An example of a stressor that will stimulate autophagy is oxidative stress. Mitochondria are the main source of free radicals within the cell and, when damaged, mitochondrial dysfunction will cause an increase in the production of free radicals and thus, oxidative stress (16). With an increase in oxidative stress, increased accumulation of the lipidated form of LC3 has been observed within the cell (17), which suggests oxidative-stress-induced macroautophagy. This will facilitate the adaptation of the cell to oxidative stress and subsequently diminish oxidative damage by an increase in macroautophagy and the degradation of the intracellular oxidized and dysfunctional mitochondria, resulting in the decrease of free radicals and thus a decrease in oxidative stress (17).

Degradation of dysfunctional mitochondria

Selective macroautophagy of mitochondria, or mitophagy, refers to the degradation of damaged or surplus mitochondria via targeting to the lysosome for destruction (18). One of the free radicals produced by oxidative stress is the highly reactive hydroxyl radical which can damage the DNA of mitochondria; ultimately resulting in mitochondrial dysfunction and further production of free radicals (3). A reduction in autophagy of mitochondria is partly responsible for the dysfunction of mitochondria in aged organisms. In livers of aged wild-type mice, an extensive accumulation of double-membrane vesicles with cargo in autophagosomes has been observed, and this has been attributed to a problem with autophagosome clearance by lysosomes, which increases with aging (19). Additionally, cells deficient in the essential Atg5 and Atg7 proteins for autophagy will accumulate defective mitochondria (4,25).

Role of macroautophagy in preventing inflammation

Oxidative damage will result in the production of reactive oxygen species (ROS), which are free radicals that can lead to intracellular damage and inflammation. An increase in ROS is induced by the inhibition of autophagy, since normal autophagic processes will remove dysfunctional mitochondria through mitophagy and thus prevent the excessive production of ROS (3,24). The inflammasome, which is an intracellular multiprotein sensor that will respond to a number of danger signals, will be induced by the introduction of foreign substances or cellular damage such as the over-production of free radicals in oxidative damage. A danger signal such as this will lead to the stimulation of an inflammatory response and the release of pro-

inflammatory cytokines (20). Autophagy will prevent inflammation by removing inflammasome components from the cell, therefore reducing the inflammatory response (21).

Studies suggest that macroautophagy and macrophage migratory inhibitory factor (MIF) are associated; inhibiting autophagy has been found to result in the secretion of MIF (9). MIF is a regulator of the immune response to inflammatory stressors, and dysregulated MIF is a key mediator of acute and chronic inflammatory processes (9). MIF will promote inflammation through the induction of pro-inflammatory family cytokines such as tumor necrosis factor (TNF), interleukin-6 (IL-6), and interleukin-1 (IL-1), and the production of these cytokines would not occur without this factor (22). Therefore, through inhibiting MIF, autophagy prevents inflammation.

Autophagy in female animals

Potential sex differences in autophagy could influence the susceptibility to inflammation and oxidative stress and thus the autophagic flux in multiple organs, including the liver. Since autophagy, inflammation and oxidative stress are linked, and females have lower inflammation and oxidative stress, differences in autophagy could explain these sex differences (12). Research has shown organ-specific effects of female sex hormones on autophagy. During the normal reproductive cycle, the endometrial wall proliferates under the influence of estradiol and progesterone; this is an anabolic process. Under these conditions, female sex hormones inhibit autophagy, a catabolic process. In the uterus, autophagic proteins like LC3 and thus, autophagy, are activated by loss of the steroid sex hormones such as estrogen and progesterone, demonstrating an inhibitory effect of these hormones on autophagy (12). Furthermore, Choi *et.*

al demonstrated that ovariectomy (loss of female reproductive hormones) induced autophagy in the uterus (29, 30).

In contrast to the uterus, the effects of female sex hormones on autophagy in the liver appear to be stimulatory. For example, Kim *et. al* observed that ovariectomy decreased AMP-activated protein kinase (AMPK) phosphorylation in the liver of OVX mice. Since the phosphorylation of AMPK promotes autophagy, their results suggest a stimulatory effect of female sex hormones on autophagy (28). Similarly, Kanazawa recently demonstrated an effect of female sex on autophagy. In response to an ovariectomy (OVX), he observed that there was a decrease in autophagic markers, initially suggesting that removal of female sex hormones would decrease autophagy. As a result of his experiment, he found that LC3-I decreased as a result of OVX, but that LC3-II did not change. However, as a result of OVX, because of the respective increase in LC3-II in relation to LC3-I, the ratio of LC3-II to LC3-I increased, indicating an induction of autophagy as a result of OVX. As Kanazawa determined, it is difficult to assess the amount of autophagy through the analysis of the LC3-II/LC3-I ratio alone, so he also determined the amount of p62 protein within the rats. The amount of p62 was found to be greater in OVX rats than the control rats, therefore indicating that hepatic autophagy was actually lowered in the OVX rats and suggesting a stimulating role of estrogen. As a result of direct injection of ovarian hormones such as B-estradiol and progesterone, the LC3-II/LC3-I ratio also increased, but the amount of p62 decreased, indicating that autophagy can be induced by ovarian hormones (13).

Autophagy in male animals

While previous studies have shown that a decrease in testosterone, which can be induced by castration, will promote autophagy and limit apoptosis in prostate cancer cells, Wang *et. al* sought to determine whether castration would play the same role in the regulation of autophagy in vivo and in vitro by utilizing Male Sprague-Dawley rats and their corpus cavernosum smooth muscle cells (CCSMCs) (15). They found that castration inhibited autophagy within smooth muscle cells by decreasing the amount of autophagosomes based on LC3-II analysis. They replaced testosterone within the castrated animal groups and found a partial improvement in autophagy (15). Much less is known about the effects of sex hormones on autophagy on the livers of male mice. Overall, sex hormones influence the process of autophagy, and there are organ-specific effects. These sex-specific differences in autophagy might explain the differences in inflammation and oxidative stress in the livers of male female mice.

Chapter 3

Materials and Methods

All animal protocols were approved by Rutgers University, and all tissues samples were harvested during previous experiments conducted at Rutgers University under appropriate IACUC protocols. The livers of male (n=6) and female (n=5) SAMP-8 strain mice of 25-weeks of age were frozen in liquid nitrogen to be prepared for homogenization. Neuroblastoma cells were obtained from Novus Biologicals and utilized as positive controls for this experiment.

Sample Preparation and Immunoblotting

The livers of the animals were taken from the freezer and homogenized to obtain a protein extract. Protein concentrations were determined on the lysates. The fluid extract was added to sample buffer to equalize the protein concentration across all animals. Equal volumes of this extract were pipetted into the polyacrylamide gel, which was exposed to an electrical current (100 Volts). After a period of 90 minutes, the proteins that had been separated were then transferred onto a nitrocellulose sheet in order to immobilize the separate proteins. The membranes were then incubated in tris buffered saline (TBS) using a non-fat milk and Tween-20 solution, and then incubated with the primary antibody. Samples were incubated overnight in the p62 primary (or 1˚) antibody in a humidified chamber set at 4˚C. The next day, the membranes were washed in TBST (which is a mixture of the tris-buffered saline and Tween-20), and then exposed to the secondary (2˚) antibody for 45 minutes at room temperature. The 2˚ antibody

solution was conjugated to horse radish peroxidase, which produced light after exposure to fluid with hydrogen peroxide (H_2O_2). After secondary antibody exposure, the membranes were once again washed with the TBST solution, and then exposed to chemiluminescent substrate (containing H_2O_2) for 5 minutes. After this incubation, the light produced was photographed using the Chemi-Doc XRS system (BioRad). After this step, the membranes were thoroughly washed with TBST, and then blocked again. The same process used for p62 was repeated for the LC3 protein. Positive control samples were used on each blot to confirm the identity of each protein band. After photographing the LC3 protein, the membranes were rinsed with water and then treated with Ponceau stain, and photographed to ensure equal protein loading in each lane of the Western blot.

Band Determination

Once the immunoblotting had been performed and the samples had undergone a Ponceau stain to determine protein amounts, we performed densitometry tests on the bands. Using Image Lab software, the brightness of each band for p62 and LC3 in each sample was determined based on the light emitted from each band. The density of the Ponceau staining was also determined for each band. The brightness of the p62 band was normalized to the density of the Ponceau band for each sample. The LC3-II/LC3-I ratio was determined by measuring the brightness of each band. This ratio was also normalized to the density of the Ponceau staining for each band. Results were further normalized to the male mice group, which was given a value of 1.

Statistics

To determine the level of autophagy within the cell, we measured both the LC3-II/LC3-I ratio and p62 density. These results were then determined for their significance using a t-test for independent samples.

Chapter 4

Results

Protein Quantification

Based on the positive control for the protein, p62 was found to be at the appropriate molecular weight at 62 kDa on the Western blot membrane (Figure 1). The top band of the Western blot membrane was used to assess the protein molecular weight, since it was closest to 62 kDa. We observed three protein bands in the positive control for p62, which could be due to post-translational modifications of the protein, or interactions with other cellular proteins like LC3 (10). Protein abundance of p62 was approximately 25% lower in females in comparison to males ($p=0.051$) (Figure 3). A Ponceau wash was done to account for potential differences in protein loading between samples. These results show that each lane of the Western blot contained similar amounts of protein (Figure 3).

Based on the representative positive control blots, both of the LC3 proteins were found at their appropriate molecular weights (6 and 14 kDa, respectively). The intensity of the LC3 bands were barely above background in our representative blots (Figure 2). Results for protein quantification were determined by taking the ratio between the LC3-II and LC3-I proteins; there was no significant difference in the LC3-II/LC3-I protein ratio between the male and female mice (Figure 4).

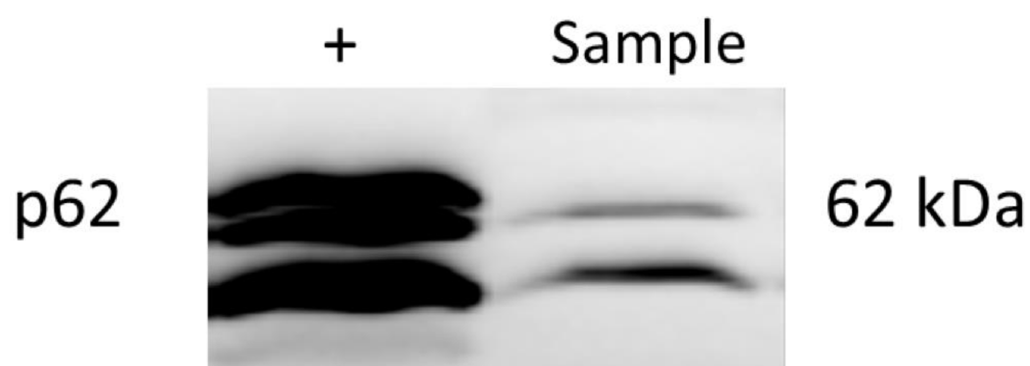

Figure 1: Western blots for p62. The first lane shows the positive control ("+") for p62 from the neuroblastoma cell line. The second lane ("Sample") shows a liver sample.

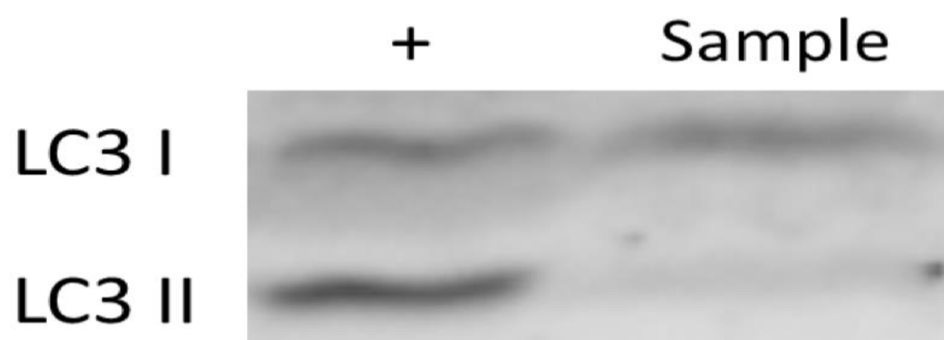

Figure 2: Western blot for LC3-II/LC3-I protein. The first lane shows the positive control ("+") for LC3-II/LC3-I from the neuroblastoma cell line. The second lane ("Sample") shows a liver sample.

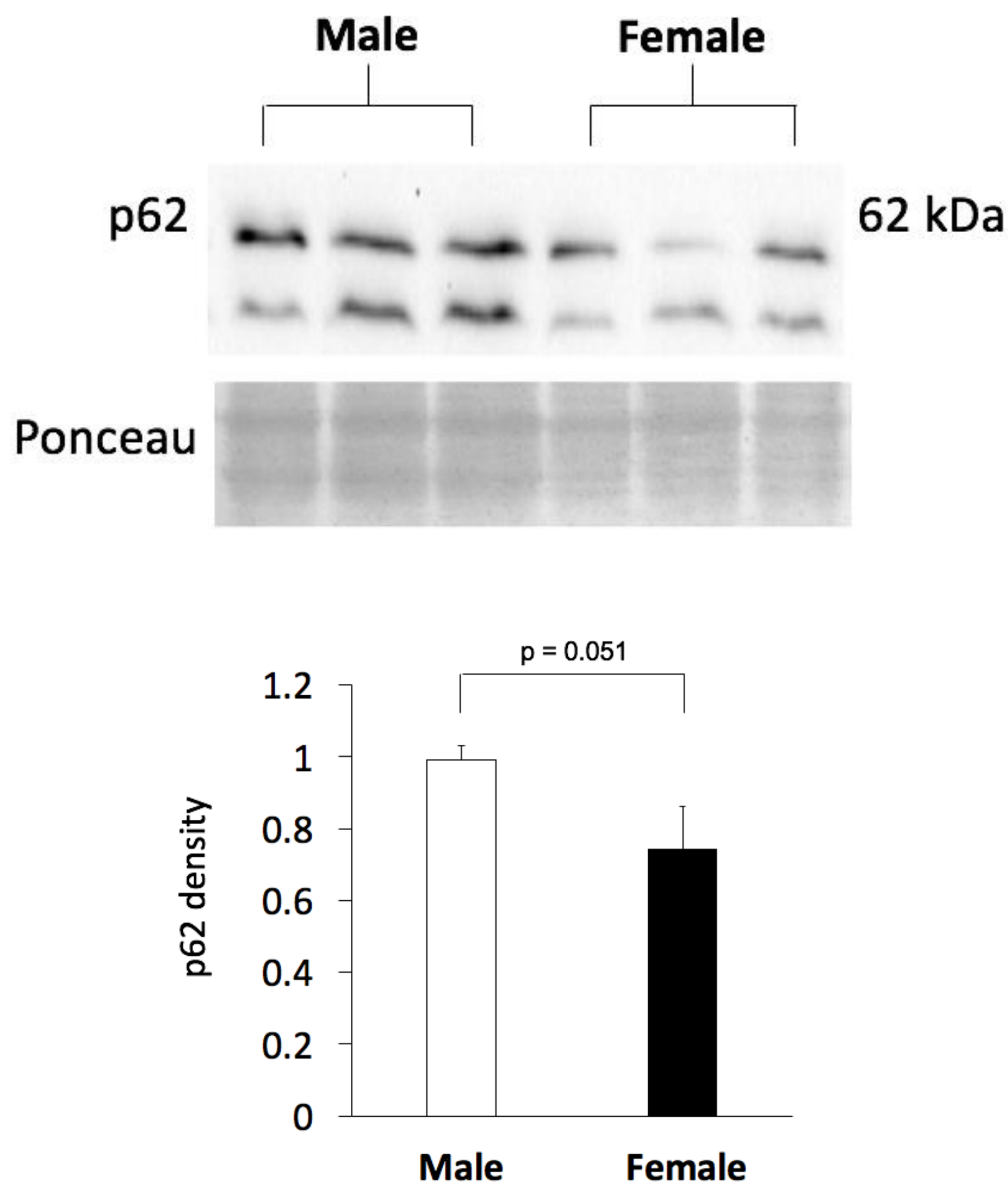

Figure 3: Representative Western blots for the p62 protein in the male and female mice (top panel). Quantitation of p62 protein abundance in males and females (bottom panel). Results are presented as the ratio of p62 brightness to the density of the Ponceau stain. Results were further normalized to the male group, which was given a value of 1. Data are expressed as means + SEM.

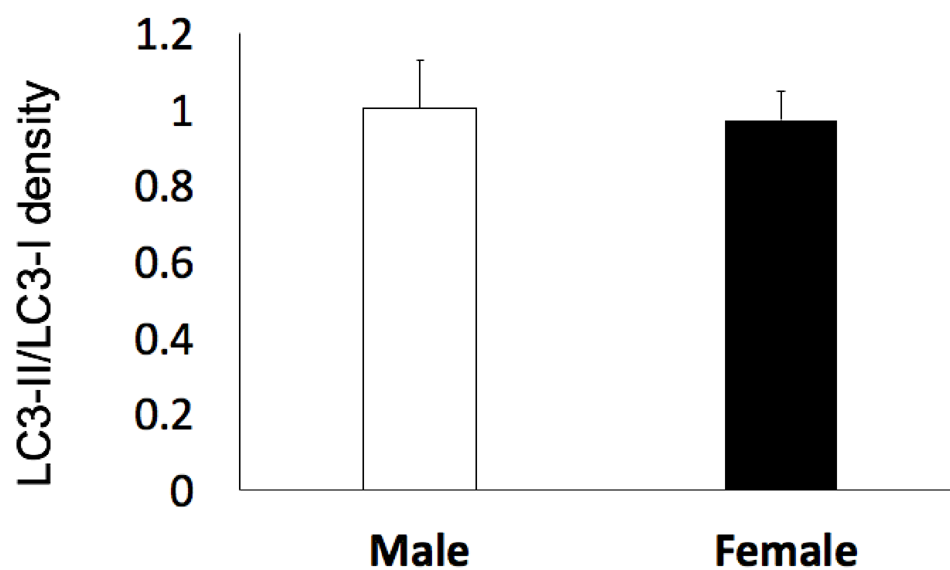

Figure 4: LC3-II/LC3-I protein ratio abundance in males and females. Results are presented as the ratio of p62 brightness to the density of the Ponceau stain. Results were further normalized to the male group, which was given a value of 1. Data are expressed as means + SEM.

Chapter 5

Discussion

In this series of experiments, we determined that the amount of p62 protein was reduced in female SAMP8 mice, suggesting a larger amount of autophagic activity in female mice. Our results are similar to the Kanazawa *et. al* paper, where he performed OVX on female mice, and made them “male-like” to observe their autophagy protein expression (13). Similar to our results, Kanazawa observed that the amount of p62 was found to be greater in OVX rats than the control rats, indicating that hepatic autophagy was actually lowered in the OVX rats, suggesting a stimulating role of estrogen in the liver (13). However, Kanazawa did not perform a male and female comparison, which would have compared the effects of OVX on autophagy to the effect of male hormones, which is why our experiment in different.

While these results are suggestive of a stimulatory role for estrogen in autophagy, some studies have shown that p62 can be a misleading marker for autophagy due to translational and post-translational modifications by lysosomal proteases (10, 11). Therefore, we also measured the ratio between the LC3-II and LC3-I proteins to determine the amount of autophagosome formation in the male and female mice. An increase in the LC3-II to LC3-I ratio typically suggests greater autophagic activity, yet there was no difference in this ratio between male and female mice.

Like p62, the regulation of LC3 protein is a dynamic process. While LC3-I will solely bind to the cytoplasmic side of the autophagosome membrane, LC3-II can bind to the cytosolic

surface and the inner membrane of the organelle (26). LC3-II on the cytoplasmic side may not necessarily be degraded in the process since it will be cleaved off by the Atg4 protein in order for the complete elongation and closure of the forming autophagosome (26), and LC3-II on the inside will be degraded by the lysosomal enzymes; thus, the same amount of LC3 could be detected even if there were differences in the rate of the autophagic process. Therefore, LC3-II can facilitate the process on the cytoplasmic side and not being consumed by autophagy (26).

Estradiol and female hormone treatment has also shown a role in inhibiting inflammation and oxidative stress. As Borras *et. al* found in their research, estrogens had a protective effect in mice and had an additional protective effect against mitochondrial free radical production (7). Similar effects were seen in the lessening of inflammation in mice. In research done by Song *et. al*, estradiol treatment in mice embryonic fibroblasts (MEFs) reduced inflammatory responses through the Nrf2 signaling pathway, which ultimately leads to the inducement of IL-10, an anti-inflammatory cytokine (35).

Our results mirror those found by these researchers. Since autophagy, inflammation and oxidative stress are linked and research has shown that females have lower inflammation and oxidative stress, differences in autophagy could potentially explain these sex differences (12). As we found in our research, we observed a higher amount of autophagy in females. In oxidative stress, mitochondria will produce peroxides, causing oxidative damage. In response to an increase in peroxides, oxidative-stress-induced macroautophagy occurs (17), which results in the decrease of free radicals and thus a decrease in oxidative stress (17). Combined with the protective effect of estrogen against mitochondrial damage found by Borras *et. al*, these results support that autophagy is linked with the oxidative stress response and the lessening of its damage in females.

Differences in autophagy could also explain the sex differences in inflammation. The inflammasome is an intracellular multiprotein sensor that will respond to a number of danger signals, such as the over-production of free radicals in oxidative damage. A danger signal such as this will lead to the stimulation of an inflammatory response and the release of pro-inflammatory cytokines (20). Autophagy will prevent excessive inflammation by removing inflammasome components from the cell, therefore reducing the inflammatory response (21). Merged with the results from Song *et. al*, these results support that autophagy is likely involved in the lower inflammatory response in women.

In conclusion, the results from this research could be used to initiate a number of different experiments related to autophagy and the differences between sexes. Our results with p62 suggested that females have higher constitutive activity of autophagy. Since autophagy inhibits oxidative stress and inflammation, the higher amount of autophagy in females could contribute to their longer lifespans. In future studies, it would be interesting to note how autophagy would affect the different sexes and their lifespan, along with the development of diseases associated with inflammation and oxidative stress.

Chapter 6

Future Directions

In this study, we primarily focused on the sex differences in autophagy to propose a link with differences in oxidative stress and inflammation. While experiments similar to this proposal have been performed before, research of these mechanisms in the liver are lacking. Studies have shown that hepatic autophagy will sense changes in protein metabolism and promote the maintenance of metabolic homeostasis (32). Sexual dimorphism is evident in liver homeostasis and metabolism (36). Differences in sex hormones are noted as some of the main disparities in the causes of the incidence of some hepatic diseases such as NAFLD (36), so it would be interesting to observe sex differences in hepatic autophagy and how it might explain sex differences in metabolism.

A future experiment could also look at the effects of the inhibiting autophagy in females to see if this affects lifespan. Among factors that will affect autophagy are caloric restriction and starvation (which was not performed in this experiment) would induce the process. Additionally, a future experiment could potentially manipulate protein levels of p62 to inhibit autophagy in females and see if this affects lifespan as well. There are pharmacological ways to do this; chloroquine is a known inhibitor of autophagy. As observed by He *et. al*, chloroquine will also affect the expression of p62 (33). Being a lysosomal inhibitor, chloroquine treatment will block the lysosomal degradation of p62 in the autophagosomes, thus causing p62 levels to increase (33). If this method works, then future experiments could start inhibiting proteins to see if this

affects autophagy as well. Genetic techniques could also be utilized to modify the p62 in an organism. Deng *et. al* observed that amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD)-linked mutations of SQSTM1/p62 will inhibit selective autophagy and specifically the Nrf2-targeted gene expression, thus disrupting the Nrf2 anti-oxidative stress response in mice (34). With increasing age, the prevalence of ALS between human men and women is almost equal, so it would be interesting to note how ALS-FTLD-linked and similar mutations would affect autophagy as an organism ages (34).

BIBLIOGRAPHY

1. Austad, S.N. Why women live longer than men: Sex differences in longevity. *Gender Medicine* **3**, 79-92 (2006).
2. Crimmins, E. M., Shim H., Zhang Y.S., Kim J.K. Differences between Men and Women in Mortality and the Health Dimensions of the Morbidity Process. *Clin Chem* **65**, 135-145 (2019).
3. Bhat A. H., Dar K.B., Anees S., Zargar M.A., Masood A., Sofi M.A., Ganie S.A. Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. *Biomedicine and Pharmacotherapy* **74**, 101-110 (2015).
4. Cuervo A.M., Macian F. Autophagy and the immune function in aging. *Current Opinion in Immunology* **29**, 97-104 (2014).
5. Bloomer S.A., Wellen K.E., Henderson G.C. Sexual dimorphism in the hepatic protein response to a moderate trans fat diet in senescence-accelerated mice. *Lipids in Health and Disease* **16**, 243 (2017).
6. Liu J., Li W., Chen R., McIntyre T. M. Circulating biologically active oxidized phospholipids show on-going and increased oxidative stress in older male mice. *Redox Biology* **1,** 110-114 (2013).
7. Borras C., Sastre J., Garcia-Sala D., Lloret A., Pallardo F.V., Vina J. Mitochondria from females exhibit higher antioxidant expression and lower oxidative damage than males. *Free Radical Biology and Medicine* **34**, 546-552 (2003).

8. Robert G., Jacquel A., Auberger P. Chaperone-Mediated Autophagy and Its Emerging Role in Hematological Malignancies. *EBSCOHost* **8**, 1260 (2019).
9. Deen N.S., Lee J.P., Harris J. Inducing and Inhibiting Autophagy to Investigate Its Interactions with MIF. *Methods in Molecular Biology* **2080**, 147-158 (2019).
10. Liu W. J., Ye L., Huang W. F., Guo L. J., Xu Z. G., Wu H.L., Yang C., Liu H.F. p62 links the autophagy pathway and the ubiquitin-proteasome system upon ubiquitinated protein degradation. *Cellular and Molecular Biology Letters* **21**, Article number: 29 (2016).
11. Puissant A., Fenouille N., Auberger P. When autophagy meets cancer through p62/SQSTM1. *American Journal of Cancer Research* **2**, 397-413 (2012).
12. Park J., Shin H., Song H., Lim H.J. Autophagic regulation in steroid hormone-responsive systems. *Steroids* **115**, 177-181 (2016).
13. Kanazawa T. Effects of Ovariectomy and Ovarian Hormone Administration on Hepatic Autophagy in Female Rats. *Journal of Nutritional Science and Vitaminology* **65**, 357-361 (2019).
14. Yao Q., Feng M., Yang B., Long Z., Luo S., Luo M., He G., Wang K. Effects of ovarian hormone loss on neuritic plaques and autophagic flux in the brains of adult female APP/PS1 double-transgenic mice. *Acta Biochimica et Biophysica Sinica* **50**, 447-455 (2018).
15. Wang X., Xu T., Xia L., Zhong S., Zhang X., Zhu Z., Chen D., Liu Y., Fan Y., Xu C., Zhang M., Shen Z., Castration impairs erectile organ structure and function by inhibiting autophagy and promoting apoptosis of corpus cavernosum smooth muscle cells in rats. *International Urology and Nephrology* **47**, 1105-1115 (2015).

16. Greco V., Longone P., Spalloni A., Pieroni L., Urbani A. Crosstalk Between Oxidative Stress and Mitochondrial Damage: Focus on Amyotrophic Lateral Sclerosis. *Mitochondria in Health and in Sickness* **1158**, 71-82 (2019).
17. Kaushal G., Chandrashekar K., Juncos L. A. Molecular Interactions Between Reactive Oxygen Species and Autophagy in Kidney Disease. *International Journal of Molecular Sciences* **20**, 3791 (2019).
18. Nichenko, A.S., Southern M.W., Tehrani K.F., Qualls A.E., Flemington A.B., Mercer G.H., Yin A., Mortensen L.J., Yin H., Call J.A. Mitochondrial-specific autophagy linked to mitochondrial dysfunction following traumatic freeze injury in mice. *Cell Physiology* (2019).
19. Zhang C., Cuervo A.M. Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. *Nature Medicine* **14**, 959-965 (2008).
20. Salminen A., Kaarniranta K., Kauppinen A. Inflammaging: disturbed interplay between autophagy and inflammasomes. *Open-Access Impact Journal on Aging* **4**, 166-175 (2012).
21. Qian S., Fan J., Billiar T. R., Scott M.J. Inflammasome and Autophagy Regulation: A Two-way Street. *Molecular Medicine* **23**, 188-195 (2017).
22. Lang T., Lee J.P.W., Elgass K., Pinar A.A., Tate M.D., Aitken E.H., Fan H., Creed S.J., Deen N.S., Traore D.A.K., Mueller I., Stanisic D., Baiwog F.S., Skene C., Wilce M.C.J, Mansell A., Morand E.F., Harris J. Macrophage migration inhibitory factor is required for NLRP3 inflammasome activation. *Nature Communications* **9**, Article number: 2223 (2018).

23. Xu, W., Zheng H., Yang R., Liu T., Yu W., Zheng X., Li B., Jiang S., Jiang L. Mitochondrial NDUFA4L2 attentuates the apoptosis of nucleus pulposus cells induced by oxidative stress via the inhibition of mitophagy. *Experimental and Molecular Medicine* **51**, Article number 140 (2019).
24. Tan P., Ye Y., Mao J., He L. Autophagy and Immune-Related Diseases. *Autophagy Regulation of Innate Immunity* **1209**, 167-179 (2019).
25. Levine B., Kroemer G. Biological Function of Autophagy Genes: A Disease Perspective. *Cell* **176**, 11-42 (2019).
26. Mizushima N., Yoshimori T., Levine B. Methods in Mammalian Autophagy Research. *Cell* **140**, 313-326 (2010).
27. Ballestri S., Nascimbeni F., Baldelli E., Marrazzo A., Romagnoli D., Lonardo A. NAFLD as a Sexual Dimorphic Disease: Role of Gender and Reproductive Status in the Development and Progression of Nonalcoholic Fatty Liver Disease and Inherent Cardiovascular Risk. *Adv Ther* **34**, 1291-1326 (2017).
28. Kim J., Jo K., Kim O., Kim B., Kang D., Lee K., Baik H., Han M., Lee S. Parenteral 17-ß estradiol decreases fasting blood glucose levels in non-obese mice with short-term ovariectomy. *Life Sci* **87**, 358-366 (2010).
29. Choi S., Shin H., Song H., Lim H.J. Suppression of autophagic activation in the mouse uterus by estrogen and progesterone. *J Endocrinol* **221**, 39-50 (2014).
30. Vasilenko P., Mead J.P., Weidmann J.E. Uterine Growth-Promoting Effects of Relaxin: A Morphometric and Histological Analysis. *Biology of Reproduction* **35**, 987-995 (1986).

31. Ueno T., Komatsu M. Autophagy in the liver: functions in health and disease. *Nature Reviews Gastroenterology and Hepatology* **14**, 170-184 (2017).

32. Ke P-Y. Diverse Functions of Autophagy in Liver Physiology and Liver Diseases. *International Journal of Molecular Sciences* **20**, 300 (2019).

33. He W., Wang B., Yang J., Zhuang Y., Wang L., Huang X., Chen J. Chloroquine Improved Carbon Tetrachloride-Induced Liver Fibrosis through Its Inhbition of the Activation of Hepatic Stellate Cells: Role of Autophagy. *Biological and Pharmaceutical Bulletin* **37**, 1505-1509 (2014).

34. Deng Z., Lim J., Wang Q., Purtell K., Wu S., Palomo G., Tan H., Manfredi G., Zhao Y., Peng J., Hu B., Chen S., Yue Z. ALS-FTLD-linked mutations of SQSTM1/p62 disrupt selective autophagy and NFE2L2/NRF2 anti-oxidative stress pathway. *Autophagy* **16**, 917-931 (2019).

35. Song C-H., Kim N., Kim D-H., Lee H-N., Surh Y-J. 17-ß estradiol exerts anti-inflammatory effects through activation of Nrf2 in mouse embryonic fibroblasts. *PLoS ONE* **14**, 1-13 (2019).

36. Kur, P., Kolasa-Wolosiuk, A., Misiakiewicz-Has, K., Wisniewska B. Sex Hormone-Dependent Physiology and Diseases of Liver. *International Journal of Environmental Research and Public Health* **17**, 1-26 (2020).

ACADEMIC VITA

Academic Vita of Gabrielle A. Aguilar
gaa5150@psu.edu

EDUCATION

2016 – Present **THE PENNSYVLANIA STATE UNIVERSITY** ABINGTON, PA
Major: B.S. Biology, Vertebrate Physiology Option
Honors: Letter, Arts, and Sciences
Abington Honors Program
Schreyer Honors College
Thesis Title: Sex Differences in Autophagic Protein Expression
Thesis Supervisor: Dr. Steven A. Bloomer
Abington College Undergraduate Research Activities (ACURA)
Project Title: Sex Differences in Autophagic Protein Expression

EXPERIENCE

2018 – Present **CVS PHARMACY** WILLOW GROVE, PA
Pharmacy Technician
- Strategically work and communicate with team members and automotive equipment to fill prescription medications
- Effectively manage processing prescriptions, taking phone calls from patients and insurance companies, patient questions, and handling medications to give to patients
- Resolve customer insurance adjudication issues by calling doctor's offices and insurance companies

Fall 2019 **THE PENNSYVLANIA STATE UNIVERSITY** ABINGTON, PA
Biology Peer Mentor
- Mentored first-year Biology students in Introductory Biology laboratory experiments and helped proctor quizzes given during lab
- Provided availability through email and in person outside of lab hours to help students with assignments

HONORS AND AWARDS

2016 – 2019 **THE PENNSYVLANIA STATE UNIVERSITY** ABINGTON, PA
Dean's List

2016 – 2018 **THE PENNSYLVANIA STATE UNIVERSITY** ABINGTON, PA
Abington Honors Program
- Mentored underneath Kevin Cannon, Associate Professor of Chemistry at Pennsylvania State University, Abington to assist the professor in advising students in Organic Chemistry

ASSOCIATION MEMBERSHIP/ACTIVITIES

2016 – 2017 **THE PENNSYLVANIA STATE UNIVERSITY** ABINGTON, PA

SHE CLUB Treasurer

- Managed meeting minutes and collaborated with the executive board during meetings to organize new events on campus
- Advocated for intersectional and feminist events happening on campus

2016 – Present **THE PENNSYLVANIA STATE UNIVERSITY** ABINGTON, PA

THON Member

- Participated in multiple fundraising events with the campus THON organization for childhood cancer through
- Attended the 2018 THON 46-Hour Dance Marathon