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Quantification of Autophagy in the Fat Cells of *Drosophila* Fed BHB Supplements

by

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for eight credits

For my family and friends,

without whom I would not be here

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ABSTRACT

The ketogenic (keto) diet has been popularized in the media. However, it has also been prescribed to children and adults with drug-resistant epilepsy (Liśkiewicz et al., 2021; Ułamek-Kozioł et al., 2019). After being on this diet, patients often saw decreased or reduced seizure occurrence (Ułamek-Kozioł et al., 2019).

While on a keto diet, the body produces ketone bodies, which may impact the diet's effect on seizure reduction (Ułamek-Kozioł et al., 2019). Some previous studies in mice have suggested that ketone bodies can increase autophagy, which positively impacts health and helps maintain cellular homeostasis (Liśkiewicz et al., 2021; Xiang et al., 2023).

While the keto diet can be a good treatment for those with drug-resistant epilepsy, it has side effects such as vomiting, abdominal pain, fatigue, and kidney stones (Xiang et al., 2023; Ułamek-Kozioł et al., 2019). Using ketone body supplements as an alternative treatment to the keto diet would avoid unfavorable side effects. It would only work as an alternative if ketone body supplements provided the same beneficial effects as the keto diet.

I analyzed the fat body cells of *Drosophila melanogaster* to determine if there were increased rates of autophagy in larvae fed a diet supplemented with BHB (ß-hydroxybutyrate - a ketone body) compared to larvae fed a control diet. I generated images using a Confocal Laser Scanning Microscope, and they were then analyzed using Cell Profiler to determine if the two treatments had statistically significant differences.

After statistical analysis (T-test) of the difference in average puncta per nucleus between the two treatments, the resulting p-value was 0.397. This p-value is greater than 0.05, indicating that the differences between the BHB and control treatments were insignificant.

INTRODUCTION

Autophagy

Autophagy is natural and vital in the processes of cell health and cell maintenance. As cells age, proteins and organelles become dysfunctional and degrade. Autophagy breaks down these cellular materials in lysosomes and reuses them to create new cell parts (Madeo et al., 2019). This process is crucial because it increases metabolic efficiency, controls quality, and kills cellular pathogens (Mariño et al., 2014). Additionally, autophagy aids in slowing aging, promoting longevity, and can help with some health issues such as seizures, stroke, and cancers (Khandia et al., 2019; Xiang et al., 2023; Ichimiya et al., 2020).

While autophagy is a regular housekeeping process, certain situations will induce an increase in it (Andreotti et al., 2020). These situations include fasting, calorie restriction, diets that cut out carbohydrates and focus on fats, and highly strenuous exercise (Andreotti et al., 2020; Kolb et al., 2021). Fasting and calorie restriction increase autophagy because the liver uses up stores, and the body then needs to recycle cell components to access more energy (de Cabo & Mattson, 2019; Andreotti et al., 2020). Exercise, which increases autophagy-related protein activity, and the switch to a no-carb high-fat diet, both increase autophagy (Andreotti et al., 2020; Kolb et al., 2021). These autophagy inducers work because they create stressful conditions for the body to be in, and autophagy helps the body adapt to them (Mariño et al., 2014; Andreotti et al., 2020). However,

since this is a stress response, if increased autophagy is observed, there may be other issues with cellular homeostasis (Liśkiewicz et al., 2021).

As cells age, autophagy decreases, leading to further degradation of cell function (Andreotti et al., 2020). Some studies have also found that a decrease in autophagy due to aging could be related to the onset of neurodegenerative disorders and some diseases because of a disruption of homeostasis (Andreotti et al., 2020; Liśkiewicz et al., 2021). Autophagy ensures cell health by maintaining adequately functioning organelles and proteomes. Without these healthy parts, the cell can die (Andreotti et al., 2020). Additionally, if autophagy is prevented, aging will occur more quickly. If autophagy is halted or disrupted, it can cause disease and upset cellular homeostasis (Liśkiewicz et al., 2021). Lack of autophagy can compromise cellular integrity and cause damage to add up, creating a pro-inflammatory state (Veldscholte et al., 2021). Autophagy can also be essential to overcoming a critical illness, and its restriction could lead to critical illness-induced organ failure (Veldscholte et al., 2021).

Autophagy can be divided into three types based on how the critical cellular parts arrive at the lysosome (Zhang et al., 2016). These three types are macroautophagy (autophagy), microautophagy, and chaperone-mediated autophagy (Zhang et al., 2016; Andreotti et al., 2020). Macroautophagy features lysosomes fusing with autophagosomes; microautophagy includes lysosome wrapping of cellular components; and chaperone-mediated autophagy includes components entering the lysosome themselves (Wang et al., 2022).

The steps in autophagy include initiation, vesicle elongation, autophagosome-lysosome fusion, degradation of autophagosome contents, and the release of these components to be used again (Zhang et al., 2016). Ultimately, these components will generate nucleotides, amino acids, sugars, ATP, and fatty acids (Li et al., 2020). These steps are further explained below.

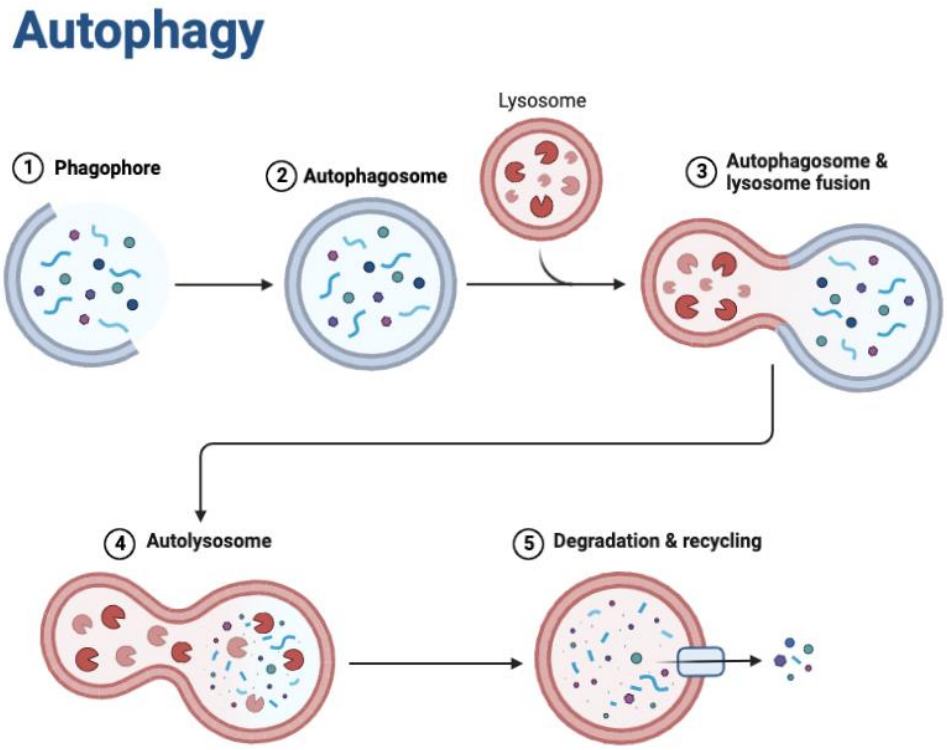

Figure 1: Steps of Autophagy. When cell components have been degraded, they become engulfed by an autophagosome. The autophagosome comes from the growth of a phagophore. Eventually, the autophagosome will fuse with a lysosome to create an autolysosome. Lastly, the contents of the autolysosome will be degraded and released to be used by the cell again (Mauvezin et al., 2014). This image was created with BioRender.com.

When proteins or organelles become dysfunctional, autophagy-related proteins help generate a membrane called a phagophore to surround and separate them from the rest of the cell (Andreotti et al., 2020; Wang et al., 2022). From here, the phagophore becomes an autophagosome when its two edges fuse, creating a double-membrane vesicle (Andreotti et al., 2020). Finally, the autophagosome fuses with a lysosomal vesicle to make an autolysosome (Andreotti et al., 2020; Mariño et al., 2014). Once fused, its contents are degraded by enzymes and released into the cytoplasm to be reused again (Li et al., 2020; Wang et al., 2022). The autophagosome will move via the microtubule network to the lysosome (Li et al., 2020; Wang et al., 2022).

Autophagy is induced by several different molecular mechanisms (some described in detail below). One mechanism is through **(de)phosphorylation** reactions run by kinases or kinase substrates. Mammalian target of rapamycin (mTOR) and autophagy-related protein Atg1, are both examples of kinases. Acetylation is also essential in some parts of autophagy and in guiding p53 and forkhead box P3 (FOXP3), transcription factors regulating autophagy (Mariño et al., 2014).

Other proteins involved in autophagy, named autophagy-related proteins (Atgs), include Atg5, Atg7, Atg12, Beclin-1, and LC3 (Mariño et al., 2014; Andreotti et al., 2020). Atg4, Atg12, and Atg16 are all necessary for assisting in forming the autophagosome and separating the dysfunctional cellular components from the rest of the cell. LC3 is associated with phagophore and autophagosome

function, and Beclin-1 is associated with the induction of autophagy through interaction with phosphatidylinositol 3-kinase (PI3K) (Andreotti et al., 2020).

ULK1/Atg1 is a complex that leads to the induction of autophagy with the help of Atgs (Li et al., 2020). ULK/Atg1 recruits autophagy-related proteins to a pre-autophagosome structure (PAS). Once the Atg proteins gather at the PAS, the membrane grows longer, creating a phagophore (Li et al., 2020).

Starvation-induced autophagy is also controlled by several units made of Atg proteins. These units are the ULK kinase core complex (with ULK1/2, Atg13, RB1CC1, FIP200, and Atg101), autophagy specific class III phosphatidylinositol 3-kinase (with VPS34, VPS15, Beclin1, and Atg14L), Atg9A trafficking system (with Atg9A, WIPI1/2, Atg2A), and the Atg12 ubiquitin-like conjugation system (with LC3A/B/C, Atg7, Atg3, and Atg4A/B/C/D) (Li et al., 2020). These units comprise the PAS (Li et al., 2020).

mTOR and AMPK

mTOR is a serine/threonine protein kinase with two different complexes, mTORC1 and mTORC2. It is a nutrient-sensing mechanism in the body and helps to maintain cellular homeostasis by responding to factors, such as nutrient availability and stress, to regulate metabolic processes. When the body has access to adequate nutrients, mTORC1 acts as a negative regulator of autophagy while allowing cell growth (Rabanal-Ruiz et al., 2017; Andreotti et al., 2020). mTORC1

is also associated with signals from AMPK that determine whether the cell will continue or limit growth (Rabanal-Ruiz et al., 2017).

Amino acids are essential for protein synthesis and, when their levels are low, protein synthesis is halted and mTORC1 no longer inhibits autophagy (Rabanal-Ruiz et al., 2017). When amino acid levels are normal, mTORC1 is present and inhibits autophagy. These fluctuations indicate that amino acids act as signals to activate mTORC1 (Rabanal-Ruiz et al., 2017).

mTORC1 controls the growth of the cell and encourages the synthesis of new proteins and other anabolic processes (Rabanal-Ruiz et al., 2017). On the other hand, it also prevents catabolic processes, including autophagy. mTORC1 regulates autophagy through the unc-51-like autophagy-acting kinase 1 (ULK1), which is necessary for amino acid-induced autophagy. It does this through binding with ULK1 and inactivating it through phosphorylation. When stress begins, mTORC1 unbinds, and ULK1 is no longer inhibited, allowing autophagy to begin (Rabanal-Ruiz et al., 2017). Once autophagy has led to increased levels of amino acids, mTORC1 activity resumes, and autophagy is halted (Rabanal-Ruiz et al., 2017).

AMPK

Adenosine monophosphate-activated protein kinase (AMPK) is a serine/threonine kinase. AMPK increases catabolic pathways, including autophagy (Wang et al., 2022). It influences rates of autophagy in several ways.

One way is through regulating ULK1 activity, which activates autophagy when certain parts are phosphorylated and induces the recruitment of autophagy-related proteins (Rabanal-Ruiz et al., 2017; Wang et al., 2022). Additionally, AMPK impacts autophagy by recruiting downstream autophagy-related proteins (Wang et al., 2022).

AMPK inhibits several mechanisms that synthesize proteins, fatty acids, and other processes (Wang et al., 2022). It is activated when there is nutrient deprivation and low energy levels, inducing autophagy to increase nutrient sources. AMPK induces this by phosphorylating specific autophagy-related proteins (Wang et al., 2022).

ATP/AMP ratio is also vital in the activation of autophagy. If this ratio decreases too much (something that can happen during exercise), AMPK will be activated (Andreotti et al., 2020). When AMPK is activated, it represses the inhibition that mTOR exhibits on autophagy, and it activates ULK1, which allows autophagy to begin (Wang et al., 2022; Mariño et al., 2014). AMPK also activates related transcription factors (like Forkhead box O), which means an increase in levels of Atgs (Andreotti et al., 2020).

AMPK and mTOR

AMPK and mTOR move in opposing ways to regulate autophagy based on energy and nutrient availability (Wang et al., 2022). AMPK encourages catabolic processes, while mTORC1 encourages metabolic processes. AMPK can

inhibit mTORC1 in several ways, one way being its phosphorylation of part of mTORC1 (Rabanal-Ruiz et al., 2017). This component acts as an upstream negative regulator of autophagy (Zhang et al., 2016).

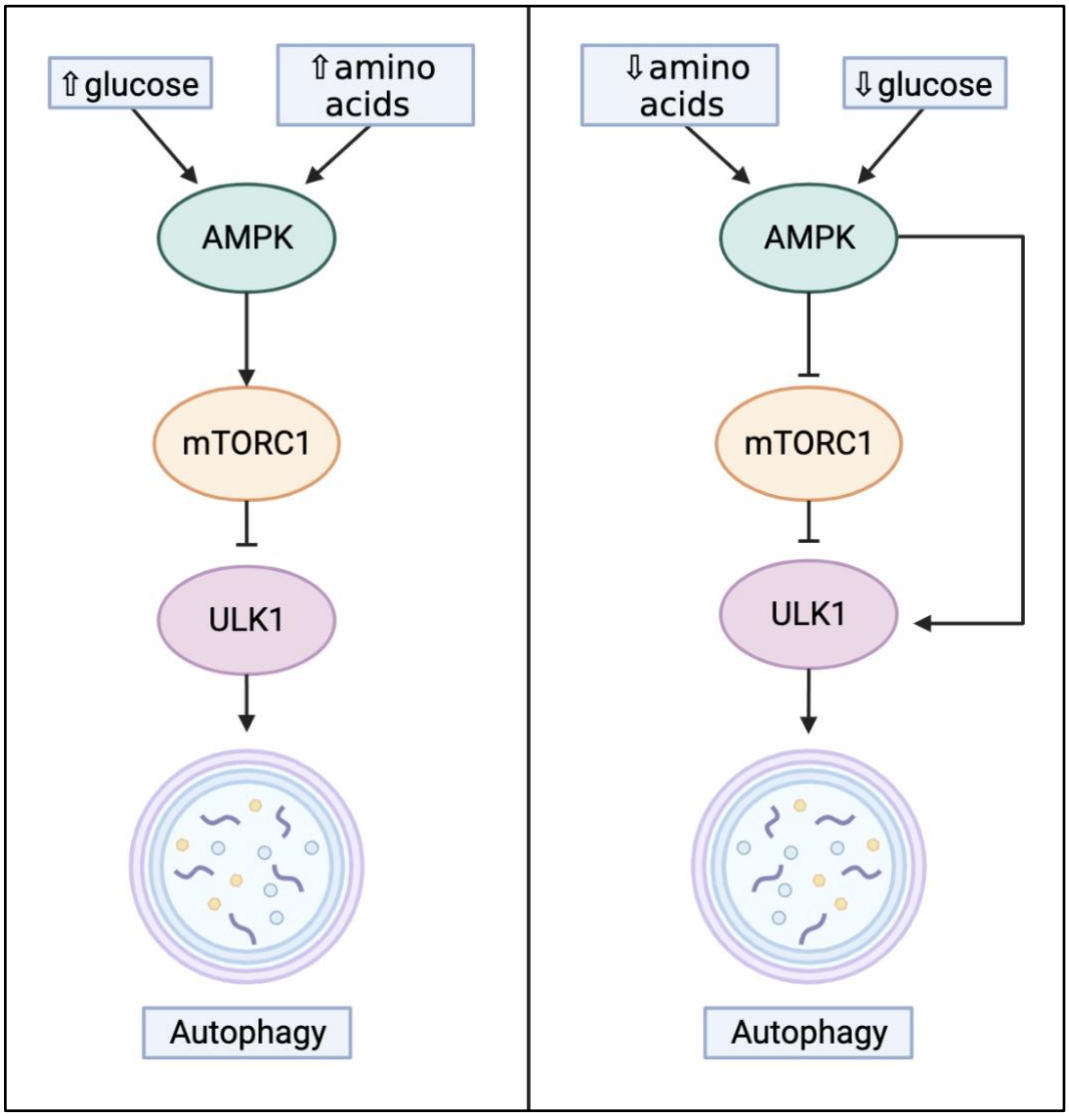

Figure 2: AMPK and mTORC1 inhibition pathway. When enough nutrients and energy are available, mTORC1 inhibits ULK1, inhibiting autophagy. However, when there is not an adequate amount of nutrients and energy, AMPK inhibits mTORC1. This means that the inhibitory effects of mTORC1 against ULK1 are inhibited, and autophagy can occur (Randall-Demllo et al., 2013; Yuan et al., 2018). This image was created with BioRender.com.

NAD+

Nicotinamide adenine nucleotide (NAD+) is thought to regulate levels of autophagy through its homeostasis. NAD+ is an electron carrier of oxidoreductase. When NAD+ is decreased, there is an increase in autophagy (Zhang et al., 2016). A decrease in ATP can induce autophagy due to decreased energy levels. NAD+/NADH helps to power the generation of ATP. If the NAD+/NADH ratios are low, or there is a low level of NAD+ (which would happen due to decreased energy levels), autophagy may be impacted (Zhang et al., 2016). More work must be done to fully understand how NAD+ regulates autophagy (Zhang et al., 2016).

Sirtuins

Sirtuins are sensors of nutrient levels and use information about those levels to regulate homeostasis. When starvation occurs, NAD+ increases, and sirtuins become activated. There are seven different sirtuin proteins located in different areas of the cell in mammals, and they are NAD+ dependent deacetylases. SIRT1 and SIRT2 are in the nucleus and cytoplasm, Sirt6 and SIRT7 are in the nucleus, and SIRT3, SIRT4, and SIRT5 are found in the mitochondria. Sirtuins control several mechanisms, including autophagy, through their deacetylation of mechanisms required for the process (Lee, 2019).

When starvation begins, SIRT1 is increased through forkhead transcriptional factor (FOXO3a). FOXO3, in turn, combines with p53 to make a

complex in relation to nutrient levels. SIRT1 can also increase due to calorie restriction, and it deacetylates p53, an autophagy inhibitor, allowing autophagy to begin (Lee, 2019). Upon increase, SIRT1 can deacetylate proteins necessary to activate autophagy, such as Atg7, Atg8, and LC3 (Lee, 2019). Autophagy that SIRT1 controls can be inhibited by REGy. REGy inhibits SIRT1 from forming complexes with autophagy-related proteins, preventing their deacetylation by SIRT1 and inhibiting autophagy (Lee, 2019).

SIRT2 also plays a role in the incidence of autophagy. However, its role is seemingly opposite to that of SIRT1. During normal conditions, SIRT2 is in a complex with FOXO1, which means FOXO1 is deacetylated and inhibits autophagy. When there is stress or starvation, this complex falls apart, and autophagy can be induced through FOXO1 interaction with autophagy-related proteins (Lee, 2019). SIRT3 also inhibits autophagy by downregulating AMPK and increasing mTORC1, an autophagy inhibitor (Lee, 2019). Lower levels of SIRT3 allow autophagy to be activated (Lee, 2019). Decreased levels of SIRT6 inhibit autophagy and lead to decreased levels of LC3 and Atg5, autophagy-related proteins (Lee, 2019).

TFEB

Transcription factor EB is a basic helix-loop-helix leucine zipper transcription factor that acts as one of the regulators of autophagy genes and, therefore, can control autophagy. TFEB phosphorylates and moves into the

nucleus when starvation is induced, activating autophagy-related gene expression (Ichimiya et al., 2020).

The Ketogenic Diet

The ketogenic diet has been popularized through media but is also used to treat various diseases and disorders. This diet focuses on a low carbohydrate, high fat, and modest protein intake (Masood et al., 2022). The diet is divided into 55% fat, 10% carbohydrate, and 35% protein (Ułamek-Kozioł et al., 2019). This diet is highly restrictive and can lead to various side effects, but it has been shown to lead to a longer life in mammals (Ułamek-Kozioł et al., 2019; Xiang et al., 2023). Interestingly, this diet mimics some effects of fasting (Lee et al., 2019).

The ketogenic diet has been used in treating diseases and disorders since 1921, starting with treating epilepsy (Masood et al., 2022). Eventually, this diet lost popularity with the arrival of other epilepsy treatments, such as antiepileptic agents (Masood et al., 2022). However, these agents are ineffective at seizure control **in about** 30% of cases (Ułamek-Kozioł et al., 2019). Patients with drug-resistant epilepsy may benefit from adopting a ketogenic diet as it may help their disorder (Masood et al., 2022). This alternative treatment to epilepsy is used globally, notably for many children (Ułamek-Kozioł et al., 2019).

The effectiveness of the ketogenic diet in human children and adults has been studied previously. In 1998, Vining et al. found that after a year on the ketogenic diet, 43% of children with drug-resistant epilepsy no longer had

seizures, 39% had controlled seizures, and 17% had no change (Ułamek-Kozioł et al., 2019). In a separate analysis of ketogenic diet use by adults, the results indicated that the efficacy rate for freedom from seizures was 13%, the reduction of seizures by half was 53%, and a reduction by less than half was 27% (Ułamek-Kozioł et al., 2019). More study is required to confirm the effects of the keto diet on epilepsy in both children and adults (Ułamek-Kozioł et al., 2019). Another example of the effects of the ketogenic diet that has been collected is that children fed on a ketogenic diet are three times likelier to have seizure freedom and six times likelier to have reduced seizures by about half. However, an analysis in line with this found no change in infants or adults (Kolb et al., 2021).

Although this diet was effective for some percentage of patients in these studies and others, some patients stopped due to the side effects of the diet. These can include lethargy, dehydration, drowsiness, vomiting, digestive issues, and mood upsets (Ułamek-Kozioł et al., 2019). Other side effects include fatigue, nausea, abdominal pain, and hunger. Even more severe side effects include hypoproteinemia, kidney stones, vitamin deficiencies, and low blood protein levels (Xiang et al., 2023; Masood et al., 2022). Other people discontinued the diet due to ineffectiveness (Ułamek-Kozioł et al., 2019). There have been attempts to modify the ketogenic diet to make it friendlier and easier to maintain (Ułamek-Kozioł et al., 2019). One solution to the side effects of the diet could be an introduced ketone body diet (Xiang et al., 2023).

In addition to treating epilepsy, a ketogenic diet positively affects other health issues. In a study on mouse models, a keto diet led mice with aging-related diseases to have a longer life, showed an increase in aged mouse motor function and cognitive function, and showed protection against brain damage (Xiang et al., 2023; Liśkiewicz et al., 2021; Veldscholte et al., 2021). Metabolic syndrome and insulin resistance are health issues the keto diet has been shown to help with (Xiang et al., 2023). More specifically, the keto diet has been used in trials to treat Type 2 diabetes, cancer, neurological disorders, and other health issues (Liśkiewicz et al., 2021). In disorders such as Alzheimer's, Parkinson's, anxiety, and depression, a ketogenic diet was found to decrease associated symptoms (Kolb et al., 2021).

The ketogenic diet induces metabolic effects like those achieved from fasting and induces metabolic starvation (Liśkiewicz et al., 2021; Choi et al., 2020). There is also evidence that a ketogenic diet can induce autophagy as fasting would (Liśkiewicz et al., 2021). Additionally, after a 1–3-week ketogenic diet, there was evidence of less oxidative stress and oxidative damage to cellular parts (Kolb et al., 2021). This diet has been shown to downregulate mTORC1 and increase AMPK activity (Xiang et al., 2023). Written in another way, it decreases the inhibition of autophagy and increases an autophagy activator.

The keto diet can induce autophagy in a variety of ways. The ketogenic diet has been found to decrease the signaling of mTORC1 through lower insulin levels, which would no longer inhibit autophagy (Liśkiewicz et al., 2021). It can

also increase AMPK, which can activate autophagy, and higher levels of fatty acids from ketosis can induce it. Other transcription factors like forkhead box O3 (FOXO3) and p53 can be increased, which are responsible for inducing the activation of autophagy genes (Liśkiewicz et al., 2021).

According to Liśkiewicz, autophagy increased in the liver of mice when fed on a ketogenic diet. LC3-II (which can mark autophagosome formation) increased when mice were fed on a ketogenic diet, indicating an increase in autophagy. They also found that SQSTM1/p63 levels, a molecule broken down during autophagy, decreased in the livers of these mice, indicating increases in autophagy (Liśkiewicz et al., 2021).

Ketosis

When the intake of carbohydrates is not restricted, the body's metabolism is centered around using glucose as energy (sourced from carbohydrates). When the intake of carbohydrates is severely restricted, the body enters a metabolic state of ketogenesis (Masood et al., 2022). The body can no longer create enough glucose, so "nutritional ketosis" begins. This leads to hepatocytes (liver cells) producing new sources of energy, ketone bodies (Masood et al., 2022; de Cabo & Mattson, 2019; Choi et al., 2020). When there is no glucose to use as energy, the body breaks down fats. This leads to fatty acids, which metabolize into acetoacetate, which is later turned into β-hydroxybutyrate (BHB) and acetone (Masood et al., 2022). Acetoacetate, BHB, and acetone are all ketone bodies that

can be used by extrahepatic tissues for energy (tissues outside of the liver) (Kolb et al., 2021). To begin the process of using ketone bodies as energy, BHB is oxidized to acetoacetate (Kolb et al., 2021). Ketosis leads the body to undergo similar processes associated with starvation (Masood et al., 2022; Choi et al., 2020). In fact, ketosis allows the body a better chance of surviving a period of starvation (Kolb et al., 2021).

Ketone Bodies

Ketone bodies (Acetoacetate, BHB, and acetone) can fuel parts of the body such as the kidneys, heart, muscle tissue, and brain (Masood et al., 2022). More specifically, ketone bodies can cross the blood-brain barrier, which can help maintain brain health (Veldscholte et al., 2021). Ketone bodies can generate more adenosine triphosphate (ATP) than glucose, and more energy can be stored in less space (Kolb et al., 2021; Masood et al., 2022). While these are beneficial effects, there is a line to be drawn between these effects and the effects that come from starvation (Veldscholte et al., 2021). Some studies have found the beneficial effects of BHB to be increased longevity and the slowing of the aging process (Xiang et al., 2023). It has also been found that BHB can bring about autophagy (Xiang et al., 2023). BHB makes up about 70% of ketone bodies. Extrahepatic tissues can use BHB and acetoacetate after they have moved into the bloodstream and through the body. The liver does not use BHB since it does not have the appropriate cellular mechanisms to break it down into energy (Xiang et al., 2023).

Ketone bodies can also act as signaling molecules that control the expression of some proteins that can impact aging (de Cabo & Mattson, 2019). Ketone bodies can also activate other signaling molecules, some of which activate autophagy, such as nicotinamide adenine dinucleotide (NAD+), which deacetylate SIRTs (Veldscholte et al., 2021).

An increase in ketone bodies can lead to a decrease in AMP/ATP ratio, an increase in NAD+ (with a decrease in NADH), an increase in radical oxygen species (ROS), and an increase in metabolic stress (Kolb et al., 2021). BHB was shown to lead to oxidative stress, inflammation, and cell damage. However, there is also evidence that BHB leads to antioxidant and anti-inflammatory activity (Kolb et al., 2021). While the beginning reactions to ketone bodies can be detrimental to cell health, it leads to more beneficial results due to the activation of adaptation and cellular defense (Kolb et al., 2021). Within this defense, we see that autophagy can be activated due to the activation of Nfr2 by increases in ROS. Increases in ROS also induce a decrease in NAD+/NADH ratio, activating sirtuins 1 and 3. These are associated with autophagy (Kolb et al., 2021). An increase in BHB levels can cause AMPK induction which regulates autophagy (Kolb et al., 2021).

BHB Supplementation

BHB supplementation may be a more accessible alternative to the keto diet. In the hopes of helping failing hearts, there has been some treatment through

the use of a racemic mixture of BHB of calcium and sodium salts or BHB esters like (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (Kolb et al., 2021). According to Finn, the study used BHB and found that ketone bodies cause some substrates to be oxidized, which in turn induces a certain kind of autophagy (chaperone-mediated autophagy) (Finn & Dice, 2005). If used for treatment, there must be a close watch on BHB levels in the human body. Typically, that level is around and below 0.5mM (Xiang et al., 2023). However, if that concentration increases too much, ketoacidosis may occur. This is a negative side effect when levels of ketone bodies are too high (Kolb et al., 2021). The body can no longer maintain the proper pH of its blood (Kolb et al., 2021).

Introducing BHB will increase levels of BHB in the body. However, ketone bodies do not stay around for very long and so it can be hard to maintain the correct concentration for therapy (Xiang et al., 2023). Using ketone body salts can also introduce too much sodium into the body, which could be avoided by using ketone esters. Poly-β-hydroxybutyrate can also be used to increase levels of BHB in the body (Xiang et al., 2023). BHB introduced into the body has been shown to affect aging positively, and further study could reveal the specific mechanisms of this (Xiang et al., 2023). Introducing BHB into *D. melanogaster* has been shown to reduce seizures (Lee et al., 2019).

CRMs

Caloric restriction mimetics could be another way to induce autophagy artificially (Madeo et al., 2019). These can work by acting as a deacetylase to some cellular proteins that lead to the activation of autophagy (Madeo et al., 2019). Some of these mimetics would also improve an organism's lifespan (Madeo et al., 2019). CRMs could include resveratrol, spermidine, rapamycin, and hydroxycitric acid (Madeo et al., 2019).

Inducing autophagy externally could be a promising treatment for various diseases (Madeo et al., 2019). CR also exhibits anti-aging effects in the body as ketone bodies do (Most et al., 2017). Additionally, some genes associated with autophagy are increased due to CR (Most et al., 2017).

Epilepsy

Epilepsy is a disorder that occurs in the brain and is chronic. A main symptom of this disorder is seizures, which means that the sufferer may have movement in different body parts that are out of their control. During this time, the person with epilepsy can become unconscious as well. There are a few kinds of epilepsy. The most common is idiopathic epilepsy, the cause of this disorder being unknown. Secondary (symptomatic) epilepsy does have a known cause, such as a brain tumor, head injury, or a brain defect (Ułamek-Kozioł et al., 2019).

Epilepsy occurs more frequently in low-income countries, with about 139 per 100,000 people having it (Ułamek-Kozioł et al., 2019) and about 80% to 90%

of people with epilepsy living there. This disorder can cause disability and is responsible for premature death and economic loss (Ułamek-Kozioł et al., 2019). Additionally, a patient with epilepsy can have a lower quality of life than someone without it (Ułamek-Kozioł et al., 2019).

Treatment for epilepsy can include options such as medicine or surgery. However, 30% of patients are not responsive to either of these options. The ideal therapy has epilepsy under control by combining two drugs (Ułamek-Kozioł et al., 2019). For those not responsive to medicine, a ketogenic diet can be prescribed and is effective for some (Ułamek-Kozioł et al., 2019).

Anticonvulsant Effects of the Ketogenic Diet

A ketogenic diet can lead to the creation of energy stores which can increase effective neurons. Additionally, this could increase the likelihood of surviving starvation or other stressful situations. It has been suggested that the ketogenic diet could influence parts of the brain to become more resistant to metabolic stress, decreasing the likelihood of seizures. Electrical excitability of the brain can also be decreased when on the ketogenic diet, which can also reduce the likelihood of a seizure. Lastly, the keto diet can also lead to synthesizing an inhibitor neurotransmitter, γ-aminobutyric acid (GABA), which helps lower neuronal excitability (Ułamek-Kozioł et al., 2019).

Drosophila melanogaster as Model Organisms

Drosophila melanogaster is an insect more commonly known as the fruit fly and is about 3mm long. Using *D. melanogaster* as a model is very convenient for experiments as their life cycle is very short. The generation time for these organisms is about ten days, including egg fertilization and the eclosion of the adult. Overall, the lifespan of *D. melanogaster* can be as long as 80 days. There are four stages to the life cycle, including embryo, larva, pupa, and adult (Fernández-Moreno et al., 2007).

The larval stage has three steps: the first instar, the second instar, and the third instar. During the first instar stage, the larva remains at the surface of the food it is cultured on and begins eating. The second instar stage sees the larva moving down into the food. Once it is old enough, the third instar leaves the food and climbs to find a suitable area to pupariate in. Pupariating takes about 24-48 hours. During this time, larval tissues are broken down and the organism undergoes complete metamorphosis (Fernández-Moreno et al., 2007).

Flies can be cultured in small vials with fly food in the bottoms. The fly food contains sucrose, agar, yeast, and propionic acid. During all this time, the organism is usually kept at around 25C. Stocks are necessary to maintain so that there are flies available for any experiments to be done. In each vial, it is necessary to have around eight females and fewer males. Each day, females produce around 100 embryos (Fernández-Moreno et al., 2007).

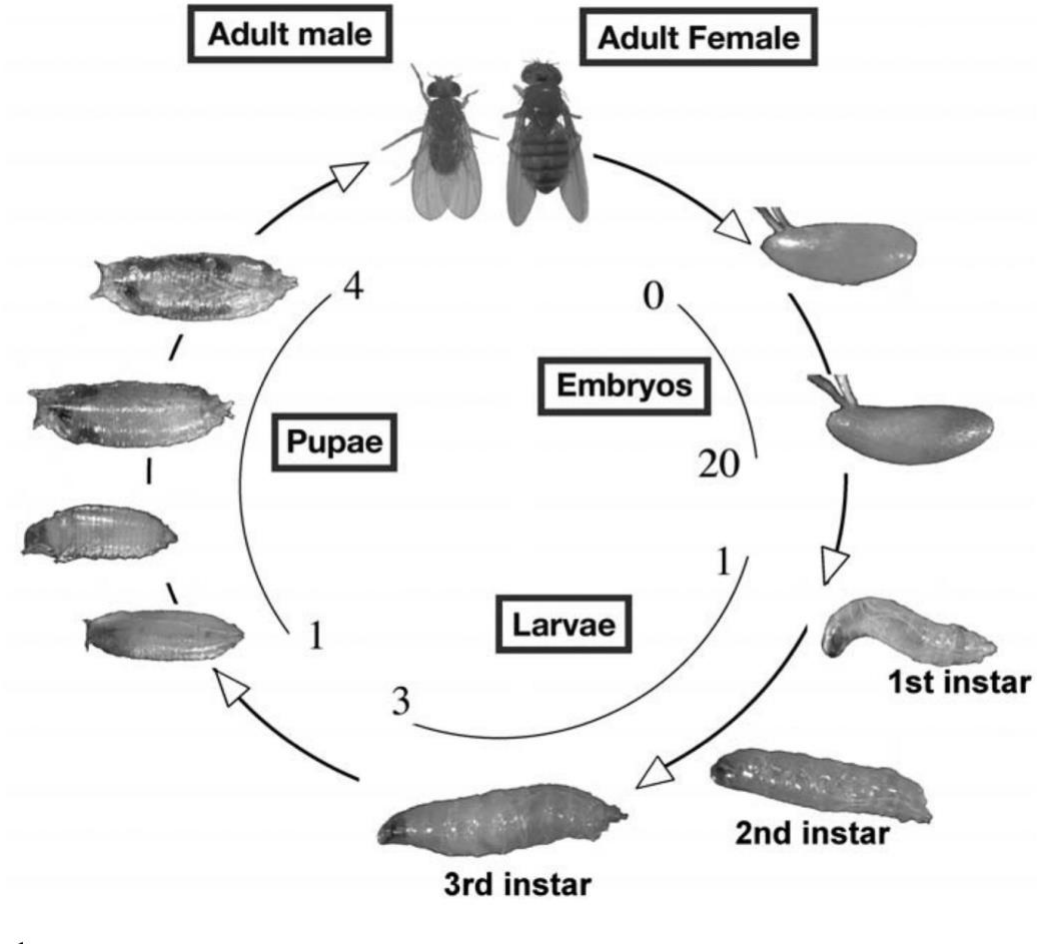

Figure 3: Life cycle of *Drosophila melanogaster*. The life cycle of *D. melanogaster* is broken into four different stages. The life cycle begins with embryos for about 20 hours. Following this, the other three stages occur over days rather than hours. The second stage sees *D. melanogaster* spend about three days as larvae, broken into three parts. *D. melanogaster* then spends around four days as pupae and emerges as an adult (Andreotti et al., 2020).

Purpose of This Study

Both ketosis and ketone bodies lead to induced autophagy and can be used to treat different diseases. However, since ketosis is reached through extreme dieting or exercise, it would be significant and helpful to find a more accessible way to induce autophagy. For example, to reduce seizures in a child with epilepsy, they may be prescribed a keto diet. However, it would be hard for a child to follow such strict guidelines for a diet. One potential alternative, and the hope for this study, is that using ketone body supplements could induce autophagy. Racemic β-hydroxybutyrate (BHB) is a potential supplement that could be administered to an organism to test its effect on autophagy (Lee et al., 2019; Kolb et al., 2021). The question is whether it would still induce autophagy even though it is not being created through ketosis but introduced instead. If the effect on autophagy is the same, it would be an excellent alternative to the side effects of the keto diet (Xiang et al., 2023).

RESEARCH QUESTION AND HYPOTHESIS

My research question is whether a ß-hydroxybutyrate supplement (BHB) will induce autophagy in the larval fat bodies of *Drosophila melanogaster*. I hypothesize that there will be a higher occurrence of autophagy in fat bodies of *D. melanogaster* larvae fed on a ketone supplement diet than those fed on a standard control diet. I predict that a higher number of autolysosomes, indicated by points of high bleaching called puncta, will be found in the larval fat bodies of *D. melanogaster* fed on BHB.

MATERIALS AND METHODS

Fly Culture Medium (Food)

Two treatments were set up for this experiment, differing only in including a BHB supplement. For the ketone supplement in this experiment, I used DL-β-Hydroxybutyric Acid sodium salt (Product number: H6502-5G, from Sigma-Aldrich). This was stored at 4°C (Zhai, 2022). Each replicate consisted of one vial of Control and one vial of BHB (experimental). Each vial was filled 1/3 of the way with Nutri-Fly Bloomington Formulation by Genesee Scientific. BF contains yellow cornmeal, agar (Type II), corn syrup solids, inactive nutritional yeast, and soy flour. The diets contained 48g carbohydrates, 1g fat, and 7.5g protein (Zhai, 2022). In addition to this food formula, BHB vials also had 2mM of sodium (R/S)-β-hydroxybutyrate (BHB). These BHB and nutrient proportions are equivalent to a past study on *D. melanogaster* autophagy rates (Zhai, 2022).

Making the Fly Food

The first step of making the fly food used in this study was to boil 1L of water and a Genesee Nutri-Fly BF 1L packet for 15 min in a large pot with a magnetic stir bar. The mixture was then mixed periodically with a spatula and the stir bar to prevent burning on the bottom of the pot. After 15 minutes, the pot was removed from the heat and cooled to 80°C. The mixture was added to a large beaker, and 10ml 10mg/ml (in Ethanol) Tegosept was added. Following the addition of Tegosept, half of the food was added to a new beaker. After the

mixture cooled to less than 70°C, 0.126g of BHB was weighed and mixed into one of the beakers of food. Marking this container as BHB was essential for distinguishing the treatments, as they look identical. Lastly, vials were filled 1/3 of the way with either BHB or Control food. All the vials were then stored in two separate containers (one for each treatment) and covered with layers of cheesecloth to prevent contamination. The vials were left until the food had solidified or overnight. Depending on their treatment, the vials were marked the next day accordingly, and each vial was then capped with cotton. If it was a BHB vial, a large black dot was added to the cotton plug, and a line was drawn on the side of the vial. Once the vials had been made, they were stored at 4°C.

Fly Stock

This study used Canton Special Stock flies kept at the Woodard lab. Stock flies were raised on a BF formula containing Tegosept and Propionic Acid. Additionally, they were kept at a consistent temperature of 25˚C.

Replicate Creation

The contents of the two treatment vials were identical other than the food in the bottom. Each set of replicates had one Control vial and one BHB vial. Seven males and eight females from stock were added to each vial. Adding this many of each ensured that there would not be too many offspring later in the vial. It was essential to prevent overcrowding as it could limit the food available to each

larva. The larvae needed unlimited food as limited food could induce starvation which would increase autophagy, skewing the study results (Li et al., 2020). After their addition, adult flies remained in these vials for around 24 hours, after which they were taken out and put into a separate vial of flies not to be used again. Keeping the adults in for 24 hours and no longer ensured that progeny would all be at the same stage in the growth cycle. The stage in the growth cycle became critical for the levels of autophagy. The target of dissection and this study were 3rd instar larvae because rates of autophagy at this stage are low (Zhai, 2022). Six days after the adults were first added, third instars appeared. One larva was taken from each treatment vial for dissection on this day.

Every replicate used new adults from CS Stock tubes that had not been previously used in replicates to include more genetic variation. Additionally, as stock tubes have adults laying eggs for anywhere from one to two weeks before they are taken out, the adult flies added to the treatment vials in this study varied in age and maturity.

Dissection

After one Control larva and one BHB larva were chosen, fat bodies were extracted from both. Fat bodies are chosen from 3rd instar larvae specifically because it is the stage before the larvae pupate, and there are low rates of autophagy (Zhai, 2022). Until it pupates, the larva eats constantly and grows its energy stores. However, when it begins to pupate, it is no longer eating, and

autophagy could increase because of the lack of incoming energy and the use of these energy stores. At the feeding 3rd instar stage, autophagy would not be induced due to developmental stages, lack of food, or lack of energy (Zhai, 2022). This is preferred because when treating the larvae with a BHB diet, it can be ensured that this is the only extra factor for inducing autophagy. All other autophagy inducers must be accounted for to be sure of the effects.

Larva were placed in depression slides within a few drops of 0.01 M Phosphate Buffered Saline (PBS) from Sigma-Aldrich. Fat bodies were then carefully extracted and placed in a few drops of PBS. Fat bodies were then prepared to be stained with 10 µM LysoTracker Red. To create the 10 µM concentration, 1 µl **o**f 1 mM LysoTracker Red DND-99 (Molecular Probes) was mixed with 99µl 1*PBS. Following this, as much PBS as possible was extracted from around the fat bodies without drying out the cells. This was to prevent the dilution of future stains. The 100 µl LysoTracker-PBS solution was then added to the depression slide on top of the dissected fat bodies in place of the PBS. The cells were left to stain for 2 minutes. After two minutes, the fat bodies were removed from the depression slide and put into a microcentrifuge tube with 30 µl of PBS for 1 minute as a rinse. A slide was prepared for the fat bodies by adding one drop of Vectashield Antifade Mounting Medium With DAPI. This allowed the nucleus to be stained and protected the Lysotracker-Red from photobleaching. Lastly, the fat bodies were added to the drop of Vectashield, and a coverslip was placed over them. After the fat bodies had been stained with LysoTracker,

extensive lengths were taken to shield the fat bodies from light. Microscope lights were kept dim and finished slides were kept in a covered box. Imaging of the fat bodies took place in a room with the lights off and a computer monitor dimmed.

The Lysotracker mixture was made in advance and stored in 100µl aliquots at 4˚C in a covered box to be more efficient.

Confocal Images

Every replicate was created on a separate day, save for two days when two replicates were done in one day. Since a BHB and Control treatment were dissected and imaged on the same day, each day, the order in which they were created and imaged was switched to ensure that images and dissecting time would not skew the data. Once the slides were created, they were imaged using a Nikon Confocal Laser Scanning Microscope. Images were taken using the DAPI and TRITC channels to show nuclei and autolysosomes. The program used to run the CLSM was Nikon Elements. Depending on how the dissection went, there could be anywhere from 3 to 15 pieces of individual fat body. An image of every piece of fat body was taken to prevent bias, and this could lead to each vial having anywhere from 3 to 15 images taken. When focusing the microscope and choosing a section of the fat body to image, only the DAPI channel was used to omit any chance of bias upon seeing the TRITC channel (as puncta are what is being studied and that can only be seen with the TRITC channel). When the image had been focused, the TRITC channel was turned on for image capture.

Figs. 4a-c show examples of confocal images of the BHB treatments. Figs. 5a-c shows examples of Control treatment confocal images. There is a great variation across treatments in the distribution of puncta across the cells.

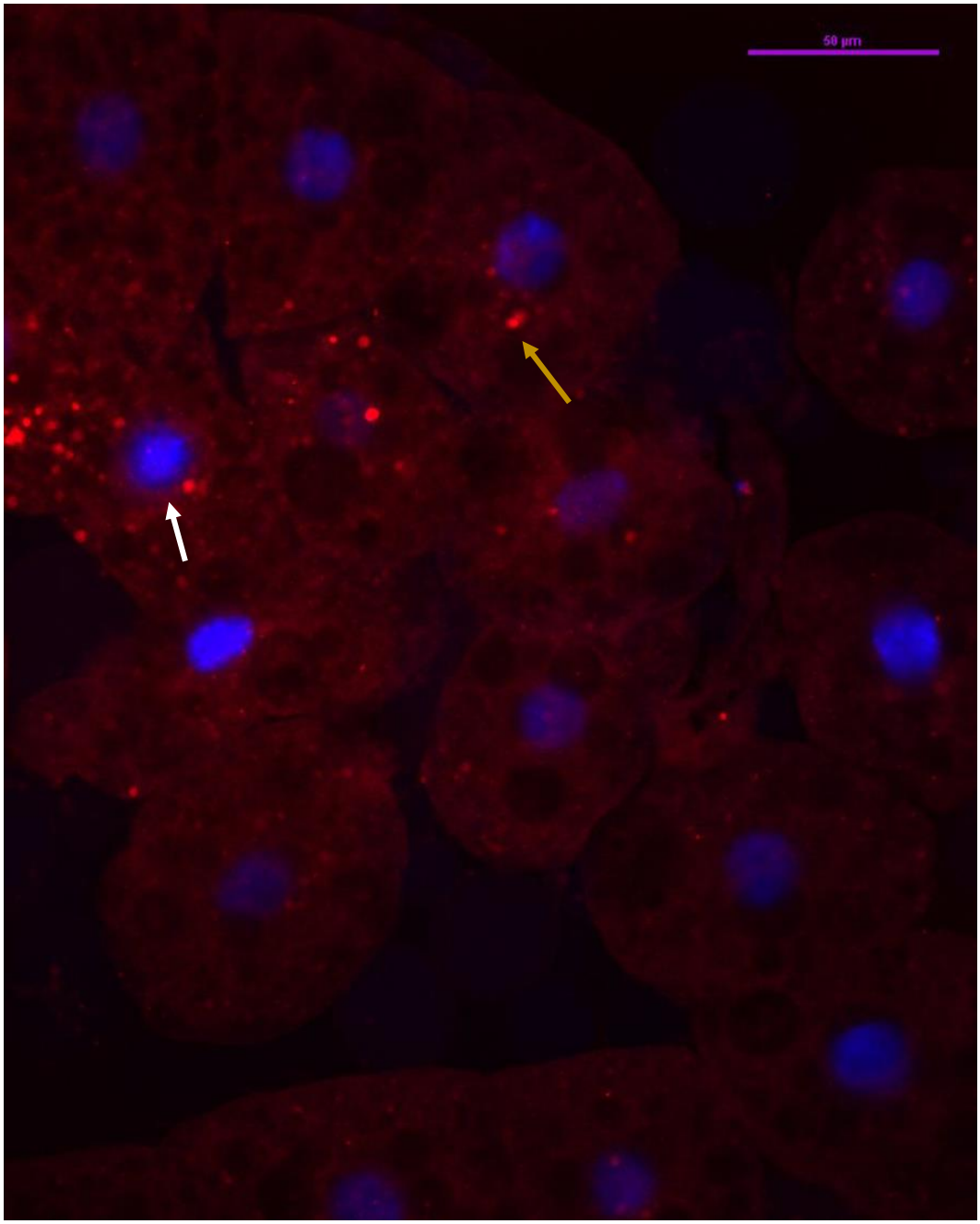

Figure 4a: Fat body cells as observed by Confocal Laser Scanning Microscopy from BHB diet-fed *Drosophila melanogaster*. Fat body cells were observed at 40x on a Confocal Laser Scanning Microscope. CLSM image obtained by exciting blue and red colors, showing nuclei (example indicated with white arrow) and lysosome/autophagy (indicated by puncta with yellow arrow). The scale bar indicates 50 µm. Nuclei are visible and there are a few clusters of puncta.

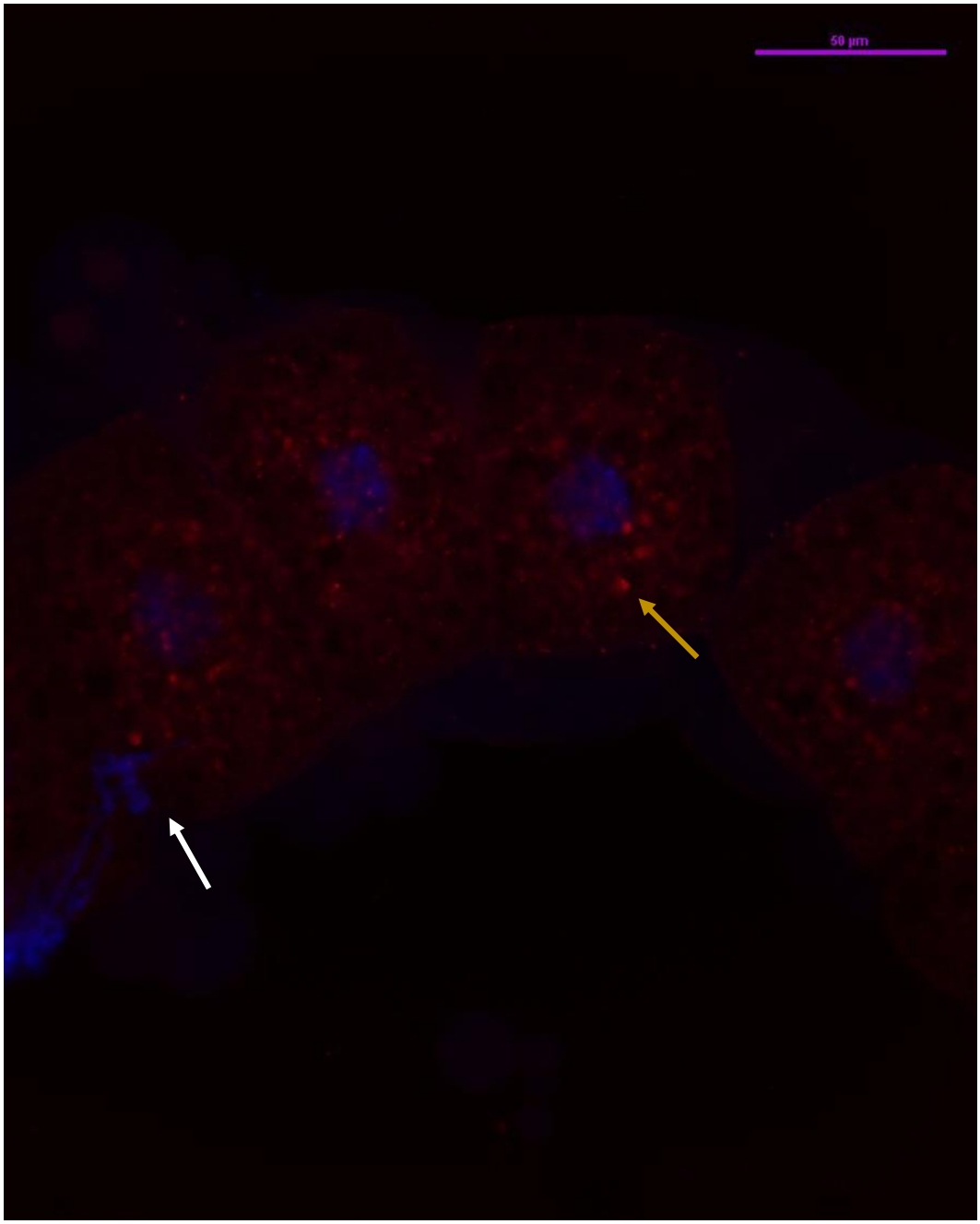

Figure 4b: Fat body cells as observed by Confocal Laser Scanning Microscopy from BHB diet fed *Drosophila melanogaster*. Fat body cells were observed using 40x on a Confocal Laser Scanning Microscope. CLSM image obtained by exciting blue and red colors, showing nuclei (example indicated with white arrow) and lysosome/autophagy (indicated by puncta with yellow arrow). The scale bar indicates 50 µm. A small group of fat body cells with nuclei and evenly distributed puncta can be seen within this image.

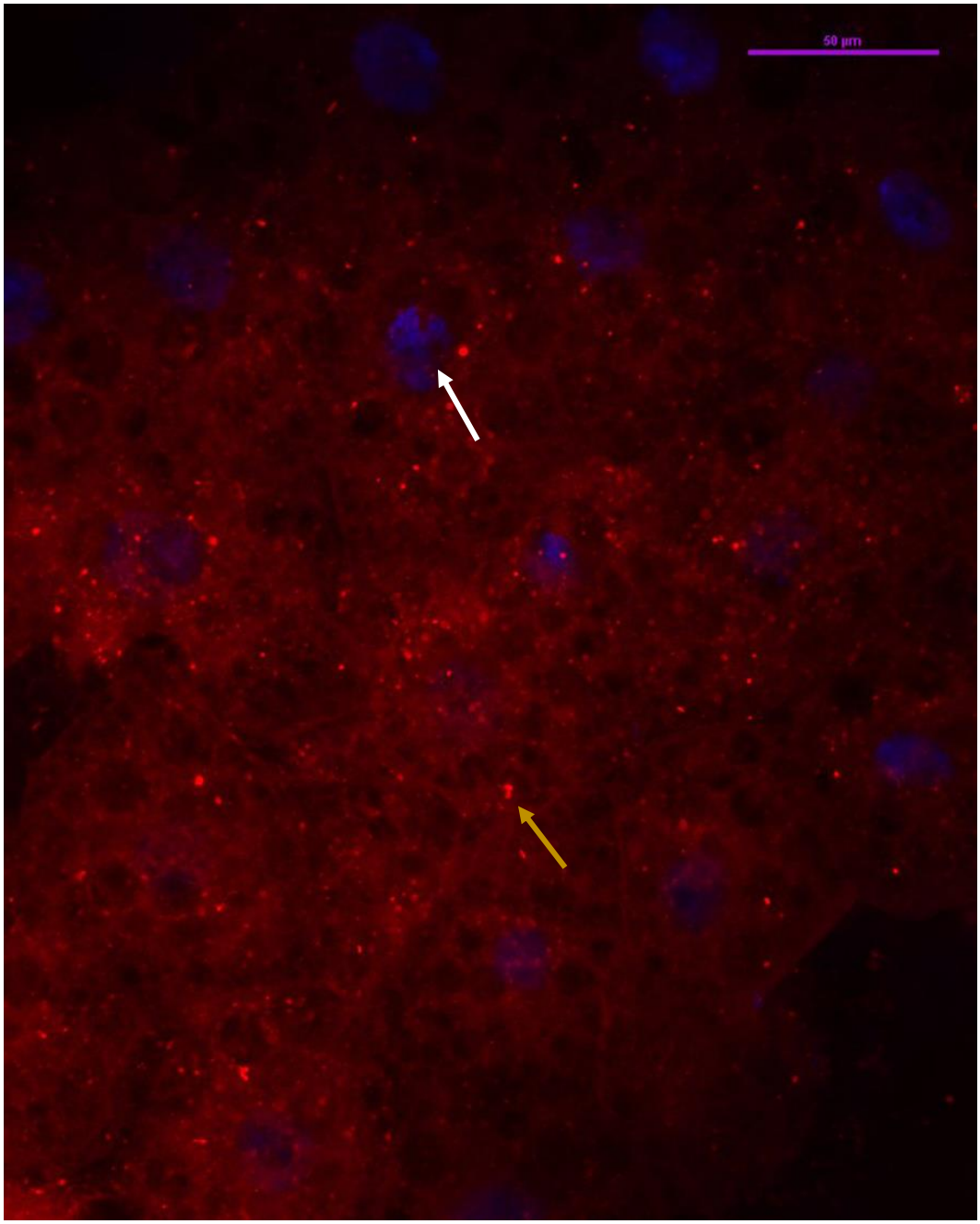

Figure 4c: Fat body cells as observed by Confocal Laser Scanning Microscopy from BHB diet fed *Drosophila melanogaster*. Fat body cells were observed using 40x on a Confocal Laser Scanning Microscope. CLSM image obtained by exciting blue and red colors, showing nuclei (example indicated with white arrow) and lysosome/autophagy (indicated by puncta with yellow arrow). The scale bar indicates 50 µm. While nuclei appear dimmer within this image, puncta are more distributed and brighter than in Fig. 4a and Fig. 4b.

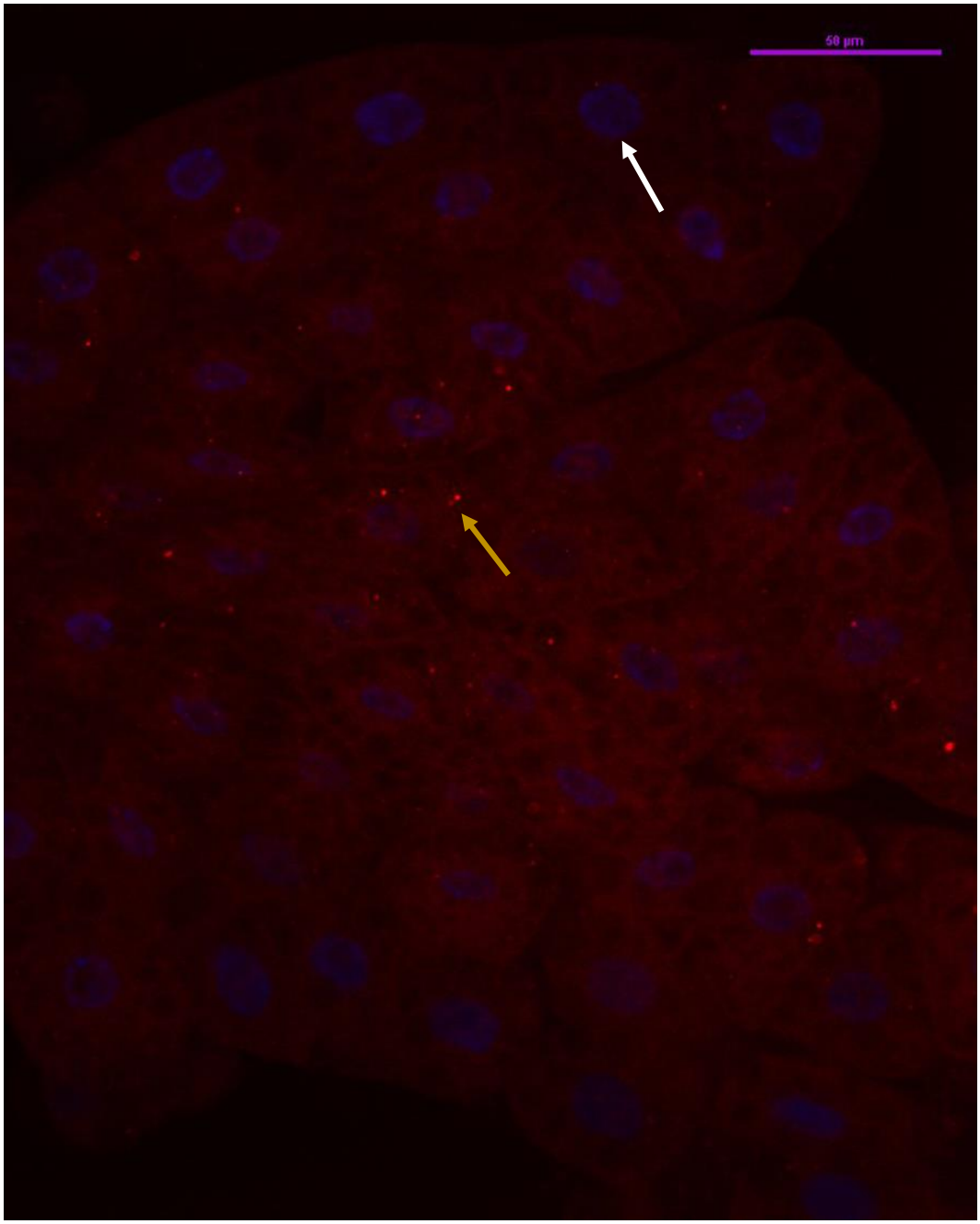

Figure 5a: Fat body cells as observed by Confocal Laser Scanning Microscopy from Control diet fed *Drosophila melanogaster*. Fat body cells were observed using 40x on a Confocal Laser Scanning Microscope. CLSM image obtained by exciting blue and red colors, showing nuclei (example indicated with white arrow) and lysosome/autophagy (indicated by puncta with yellow arrow). The scale bar indicates 50 µm. Within the fat body, nuclei can be seen faintly, and puncta can be seen distributed within a few cells.

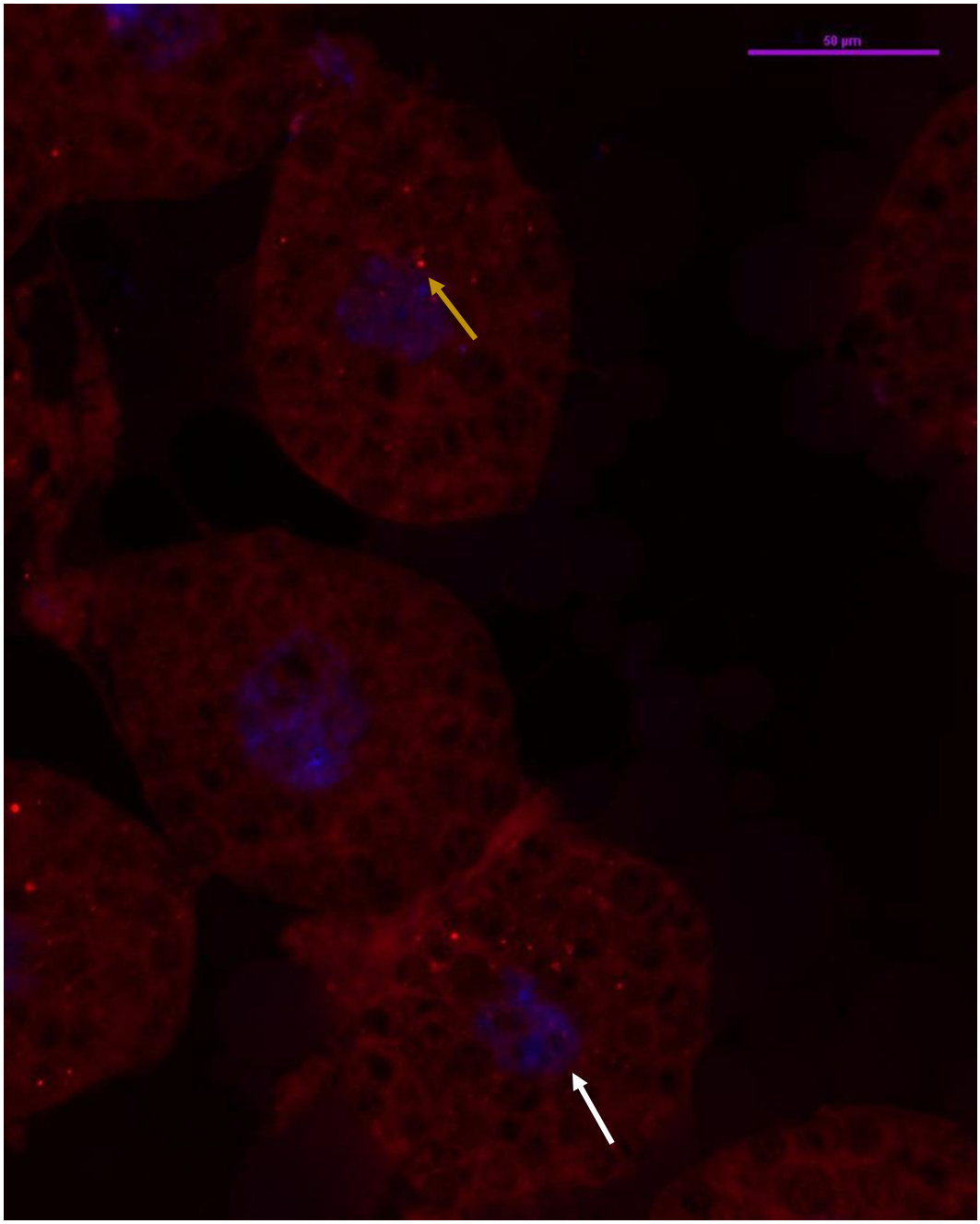

Figure 5b: Fat body cells as observed by Confocal Laser Scanning Microscopy from Control diet fed *Drosophila melanogaster*. Fat body cells were observed at 40x on a Confocal Laser Scanning Microscope. CLSM image was obtained by exciting blue and red colors, showing nuclei (shown by white arrow) and lysosome/autophagy (indicated by puncta with yellow arrow). The scale bar indicates 50 µm. Fat body cells in this image are not clustered together as in the past images. This could be due to the cells being pulled apart during dissection.

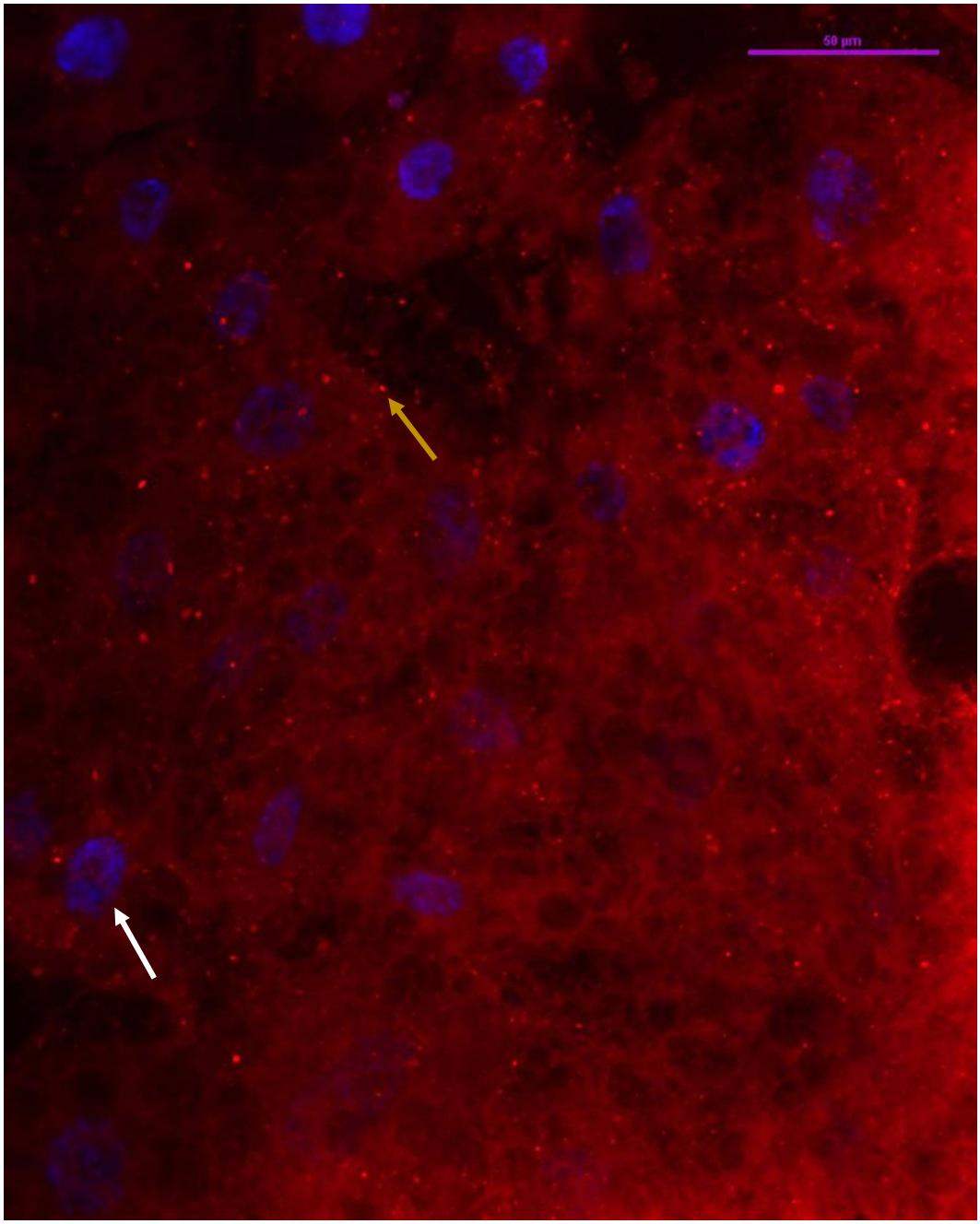

Figure 5c: Fat body cells as observed by Confocal Laser Scanning Microscopy from Control diet fed *Drosophila melanogaster*. Fat body cells were observed at 40x on a Confocal Laser Scanning Microscope. CLSM image obtained by exciting blue and red colors, showing nuclei (indicated with white arrow) and lysosome/autophagy (indicated by puncta with yellow arrow). The scale bar indicates 50 µm. Cells in this image are clustered and nuclei can be seen near the top of the image. Puncta are distributed evenly within cells, unlike Figure 5a.

Quantification

To quantify rates of autophagy between the Control and BHB treatment, images of the fat body cells with the DAPI and TRITC channels were analyzed. DAPI revealed the nuclei found in each image. TRITC stains acidic organelles, so the images using this channel revealed autolysosomes. In the images, they appear as bright red puncta. The presence of autolysosomes indicates the presence of autophagy.

Cell Profiler, an image processing program from the Broad Institute, was used to analyze the images. I hand-counted all the nuclei in each image since the program was overestimating nuclei. However, CellProfiler counted every punctum in each image using a preset threshold that did not overestimate puncta. The same threshold was applied to all images to avoid bias.

When counting nuclei, a few images with background staining made them hard to identify. When this happened, I turned the image's brightness up to find the nuclei. However, I used this strategy sparingly because it could throw off the ratio of puncta per nucleus. The brightness of the DAPI images I analyzed was set through Cell Profiler. I was concerned that if I turned up the brightness on a DAPI image, I would count more nuclei than the program would puncta (since the brightness for puncta would not be turned up). If this were the case, it would lead to lower numbers of puncta per nucleus. I did not choose to do this to either BHB or Control specifically.

Once the data had been collected on the nuclei and puncta, averages could be calculated. There were 20 replicates, each containing one individual larva fed on BHB and one fed on the Control diet. For every individual, multiple pictures were taken, but for analysis, only three images of nuclei and puncta were analyzed. These images were randomly selected using a random letter generator.

The average number of puncta per nucleus across the three images from one individual were averaged together. Once the average for each individual had been calculated, individual values in each group were averaged to come up with two final numbers. These numbers were the average puncta per nucleus in the BHB treatment and the average puncta per nucleus in the Control treatment.

Once the final numbers had been obtained, I ran a T-test on the averages to find if there was any statistical significance between the two averages.

RESULTS

The two experimental groups considered in the results were the Control and BHB groups. The control group was fed standard Control fly food, while the BHB group was fed a β-hydroxybutyrate (BHB) supplement. The two groups differed in no other ways.

When fed on a β-hydroxybutyrate supplement versus a Control diet, there is a slight increase in levels of autophagy found in *Drosophila melanogaster* fat bodies. Although there is a slight difference (as shown in Fig. 6a), it is not statistically significant ($t=0.857$, $df=38$, $p=0.397$). The null hypothesis, that there should be no difference in rates of autophagy between the two treatments, cannot be rejected, and we cannot conclude that rates of autophagy increase with BHB supplementation.

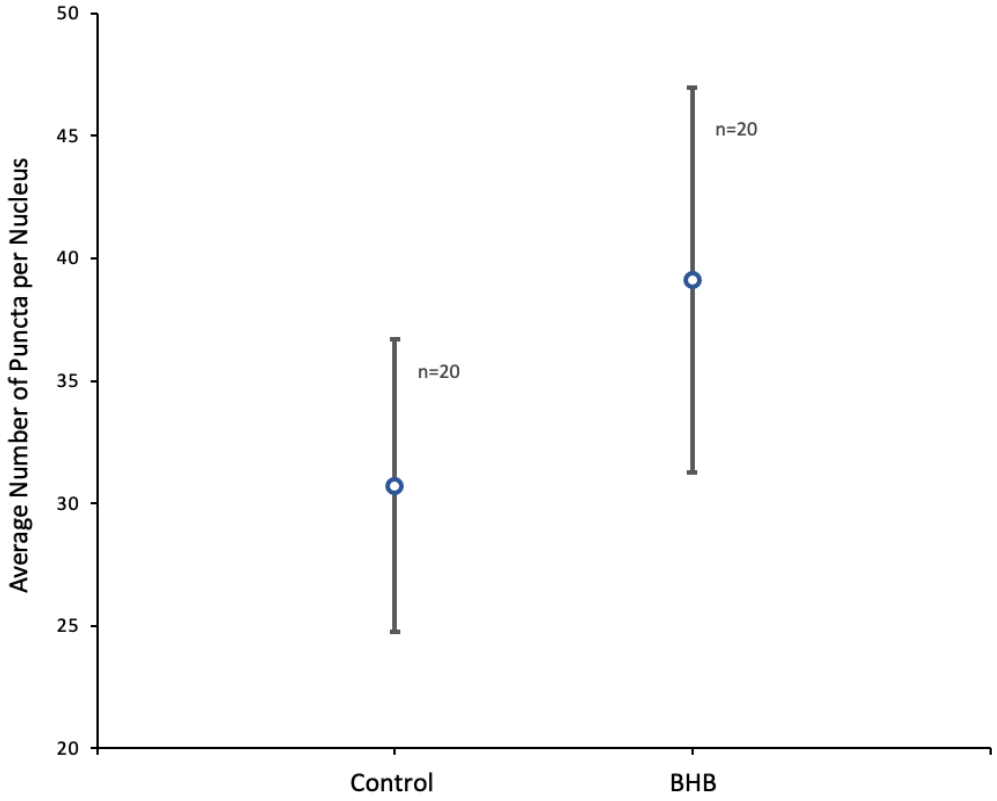

Figure 6a: Mean ± 1SE of nucleus and puncta ratio between the Control and BHB supplement groups found in Drosophila melanogaster larval fat bodies

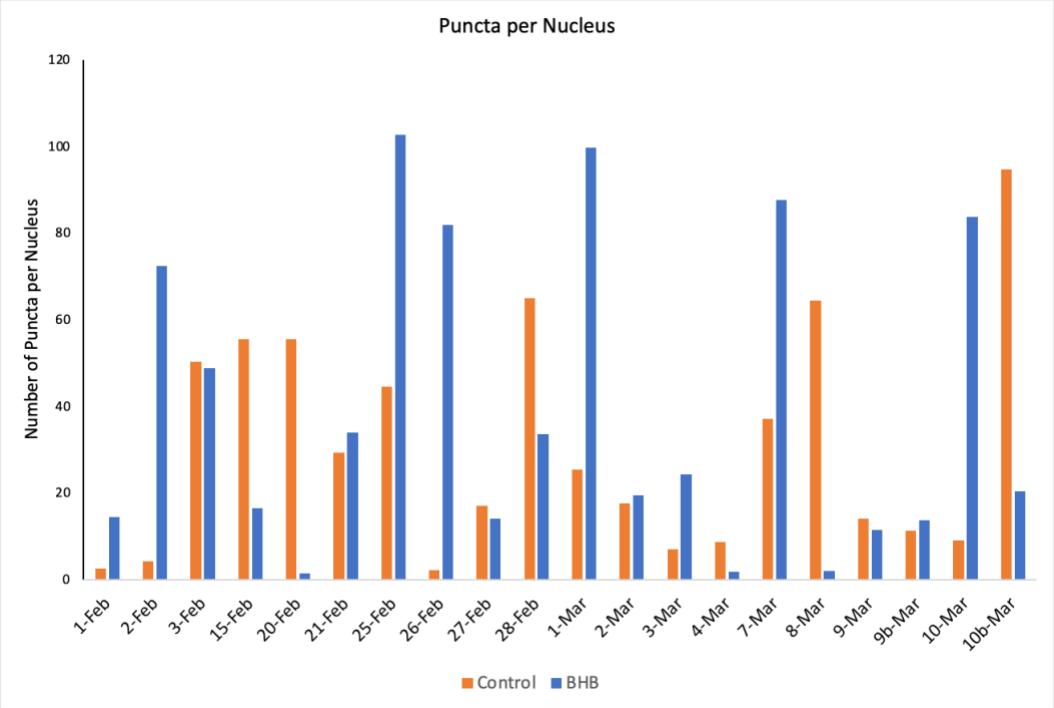

Figure 6b: Bar graph showing the difference in the average puncta per nucleus between BHB and Control individuals for each replicate. The distribution shown above shows the incredible variation in each individual in average puncta per nucleus across 3 images for each treatment over 20 replicates.

DISCUSSION

According to the statistical data and the results, there is no significant difference in rates of autophagy between the Control and the BHB diet (Fig. 6a). From this, we cannot refute the null hypothesis that a BHB supplement induces autophagy at the same rate as a regular diet. Significant variation is found within each replicate and treatment; even within the same individual, there was much variation (Fig. 6b). This variation could be due to various factors, but one of them could be the use of Lyso-Tracker Red. Lyso-Tracker Red is not a lysosome-specific stain, as it labels any acidic spherical granules (Chikte et al., 2014). Despite this limitation, Lyso-Tracker Red is still actively used to observe autophagy via the lysosome (DeVorkin & Gorski, 2014; Mauvezin et al., 2014). Lyso-Tracker Red is also less expensive than other alternatives for autophagy tracking (Chikte et al., 2014).

There are several ways to detect autophagy due to the double membrane and make up of the autophagosome (Mauvezin et al., 2014). These parts can act as markers for detecting autophagy (Mauvezin et al., 2014). Instead of using LysoTracker-Red, other autophagy markers could be utilized, such as Transmission electron microscopy (TEM) and fluorescence microscopy (Mauvezin et al., 2014). TEM allows the user to view high-resolution cellular components in an autophagosome or autolysosome. Fluorescent microscopy can be used to see the puncta of Atg8 (a protein necessary for autophagy) in several ways. An antibody that identifies Atg8a or tagged reporter transgenes, such as

mCherry-GFP-Atg8a and *mCherry-Atg8a*, could be used to track autophagy as well (Nagy et al., 2013; Mauvezin et al., 2014).

The Woodard lab has a stock of *pmCherry-Atg8a*, a transgenic *D. melanogaster* line (Mauvezin et al., 2014). In this line, mCherry is fused to ATG8a, which associates with the membrane of autophagosomes (Woodard, C., Personal communication). Using this stock of flies would have been an excellent and seemingly more accurate way to observe and quantify autophagy in *D. melanogaster* cells. However, when viewing these cells under fluorescent microscopy, it appeared that the *mCherry* was tagged to the nuclei rather than the Atg8a on lysosomes. This stock of flies was very old, spanning many generations. Because of this, a mutation could have been introduced that caused *mCherry* to no longer fuse to *Atg8a*. Due to these circumstances, the most viable option to track autophagy during my thesis period was through the use of LysoTracker-Red.

One way that I increased the quality of staining in this study was through the extensive development of experimental methods. I tried a variety of ways of staining fat bodies. Lyso-Tracker Red is associated with photobleaching, so much care was taken to shield the samples from light during the staining and imaging process. Additionally, I went from imaging the fat bodies on an epifluorescent microscope to imaging them on a Confocal Scanning Laser Microscope. This improved the quality and allowed more layers of the fat body to be captured in the image.

The Woodard and Tanner labs are both currently testing the differences in rates of autophagy between a BHB supplement diet and a Control diet. When using Atg8a-11/Atg8a-1 as autophagy biomarkers, the lab found increased autophagy of BHB-fed *D. melanogaster* fat body cells (Zhai, 2022). Additionally, there is currently ongoing analysis of the presence of autophagy between the fat bodies of BHB-fed *D. melanogaster* and Control fed *D. melanogaster* using TEM (Woodard, C., Personal communication). These studies use the same treatments as the treatments in this study, the only difference between those studies and this one is how autophagy is being observed/studied. Interestingly, preliminary results also indicate a slight but statistically significant developmental delay in BHB-fed *D. melanogaster* (Tanner, G. (2023) Unpublished findings).

Although the results of this study were not statistically significant, they are interesting to consider in light of these other current studies on autophagy concerning BHB supplements. Perhaps one reason statistical differences were found in other studies and not in this one is due to a method error in this study's experimental design. Since Lyso-Tracker Red does not stain lysosomes specifically, it may not be an accurate enough way to quantify autophagy in fat body cells. Additionally, due to photobleaching, there could be less puncta being counted than exists.

In Figure 6a., here is the slightest visible increase in autophagy in BHB-fed *D. melanogaster* fat bodies. Although not statistically significant, it is encouraging and provides ideas for future studies. One idea for the future would

be to use several different autophagy markers and see how the quantification results differ. Those results could explain the variation between this study and those mentioned in the Woodard/Tanner labs.

I am also interested in trying to induce autophagy with a different cellular mechanism. Some mechanisms to target could be mTORC1, sirtuins, or AMPK. All of these regulate autophagy in some way, and theoretically, being able to manipulate them could lead to its increase. For example, rapamycin is an inhibitor of mTORC1, and using it as a treatment would block mTORC1 inhibition of autophagy and allow it to increase (Chikte et al., 2014). Lastly, I would like to test different levels of BHB in the *D. melanogaster* diet to see how that would impact rates of autophagy. In humans, if there is too high a level of ketone bodies in the body, ketoacidosis can occur, negatively impacting the pH of the blood (Kolb et al., 2021). Knowing how levels of BHB impact autophagy would be critical for that reason.

Learning the mechanisms of why a ketogenic diet works in treating epilepsy is vital for all adults and children it impacts. Finding an alternative that can induce the same effects as the keto diet would allow children with epilepsy the chance to eat a regular diet and avoid the adverse side effects associated with the keto diet. While no statistically significant conclusions were taken from this study, how it fits into the larger narrative of other studies on BHB-induced autophagy is intriguing and promising.

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APPENDIX

Control					
	Subset	Nuclei	Puncta	Nuclei/Puncta	Average Nuclei/Puncta per Individual
1-Feb	F	4	26	6.5	
	M	4	2	0.5	
	O	12	6	0.5	2.5
2-Feb	C	31	355	11.5	
	D	32	22	0.7	
	E	20	5	0.3	4.1
3-Feb	B	7	10	1.4	
	D	10	1270	127.0	
	A	8	178	22.3	50.2
15-Feb	F	15	311	20.7	
	M	17	344	20.2	
	O	6	753	125.5	55.5
20-Feb	E	13	365	28.1	
	F	9	707	78.6	
	I	10	595	59.5	55.4
21-Feb	C	9	2	0.2	
	E	6	64	10.7	
	G	11	848	77.1	29.3
25-Feb	G	19	178	9.4	
	H	16	1312	82.0	
	K	10	422	42.2	44.5
26-Feb	D	14	82	5.9	
	F	16	6	0.4	
	J	17	3	0.2	2.1
27-Feb	A	44	1058	24.0	
	B	32	188	5.9	
	D	45	940	20.9	16.9
28-Feb	B	8	174	21.8	
	C	10	329	32.9	
	E	9	1260	140.0	64.9
1-Mar	F	12	233	19.4	
	H	18	759	42.2	
	K	13	187	14.4	25.3
2-Mar	E	19	111	5.8	
	K	16	724	45.3	
	O	32	46	1.4	17.5
3-Mar	D	32	360	11.3	
	H	53	39	0.7	
	I	33	303	9.2	7.1
4-Mar	D	16	281	17.6	
	E	7	27	3.9	
	F	20	93	4.7	8.7

7-Mar	B	37	1165	31.5	
	E	23	1562	67.9	
	J	9	107	11.9	37.1
8-Mar	C	36	3546	98.5	
	A	16	647	40.4	
	J	35	1906	54.5	64.5
9-Mar	D	17	426	25.1	
	E	23	365	15.9	
	L	16	18	1.1	14.0
9b-Mar	B	14	26	1.9	
	C	23	632	27.5	
	I	14	60	4.3	11.2
10-Mar	F	29	524	18.1	
	G	42	325	7.7	
	L	19	19	1.0	8.9
10b-Mar	L	19	141	7.4	
	D	13	3441	264.7	
	H	25	295	11.8	94.6
				Average puncta/nucleus	30.7
				Standard Deviation	26.3
				Standard Error	5.4
				n	24

Table 1a: Raw data from CellProfiler/quantification. The data above shows the puncta/nucleus from the individual subsets from each Control replicate and the average of every treatment.

BHB					
	Subset	Nuclei	Puncta	Nuclei/Puncta	Average Nuclei/Puncta per Individual
1-Feb	J	18	337	18.7	
	N	12	1	0.1	
	P	22	532	24.2	14.3
2-Feb	C	4	0	0.0	
	D	14	1530	109.3	
	E	15	1615	107.7	72.3
3-Feb	A	21	41	2.0	
	D	13	842	64.8	
	H	14	1113	79.5	48.7
15-Feb	C	16	87	5.4	
	F	21	183	8.7	
	K	13	458	35.2	16.5
20-Feb	B	30	47	1.6	
	C	20	20	1.0	
	E	12	17	1.4	1.3
21-Feb	A	9	434	48.2	
	C	5	35	7.0	
	K	9	417	46.3	33.9
25-Feb	D	7	160	22.9	
	F	9	1623	180.3	
	A	15	1574	104.9	102.7
26-Feb	I	12	595	49.6	
	E	17	107	6.3	
	F	10	1895	189.5	81.8
27-Feb	A	12	42	3.5	
	F	12	12	1.0	
	I	12	452	37.7	14.1
28-Feb	D	9	744	82.7	
	H	15	69	4.6	
	J	13	173	13.3	33.5
1-Mar	A	10	502	50.2	
	B	15	1411	94.1	
	C	23	3554	154.5	99.6
2-Mar	A	20	154	7.7	
	B	19	253	13.3	
	F	18	667	37.1	19.4
3-Mar	A	27	1009	37.4	
	E	17	154	9.1	
	H	20	526	26.3	24.2
4-Mar	B	24	21	0.9	
	F	13	43	3.3	
	H	15	14	0.9	1.7

7-Mar	I	10	1306	130.6	
	J	11	1248	113.5	
	L	12	226	18.8	87.6
8-Mar	B	24	34	1.4	
	C	12	33	2.8	
	D	40	59	1.5	1.9
9-Mar	A	15	120	8.0	
	H	41	107	2.6	
	J	24	567	23.6	11.4
9b-Mar	A	7	20	2.9	
	K	17	423	24.9	
	M	16	212	13.3	13.7
10-Mar	G	25	4499	180.0	
	H	21	1275	60.7	
	K	18	191	10.6	83.8
10b-Mar	C	13	31	2.4	
	N	12	650	54.2	
	T	31	132	4.3	20.3
				Average puncta/nucleus	39.1
				Standard Deviation	35.2
				Standard Error	7.9
				n	20

Table 1b: Raw data from CellProfiler/quantification. The data above shows the puncta/nucleus from the individual subsets from each BHB replicate and the average of every treatment.