5-2024

The anti-diabetic drug metformin disrupts feeding and sleeping behaviors in Drosophila melanogaster.

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The anti-diabetic drug metformin disrupts feeding and sleeping behaviors in *Drosophila melanogaster*

By

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Submitted in partial fulfillment of the requirements for Graduation *summa cum laude*

and

for Graduation with Honors from the Department of Biology

University of Louisville

May 2024

Abstract

Dimethylbiguanide, also known as metformin, is the single most prescribed oral treatment for non-insulin dependent diabetes mellitus, or type 2 diabetes, in Western countries. The primary mechanism of action that metformin acts through is the activation of AMP kinase, an important regulator of energy homeostasis. While the anti-diabetic effects of metformin are well documented, its effects on feeding and sleeping behaviors are not well characterized. Using the model organism *Drosophila melanogaster*, the mean daily quantity of food consumed was measured and compared between groups treated with several dosages of metformin. Feeding interactions such as meal frequency and length were also measured using the Fly-to-Liquid-food Interaction Counter (FLIC). Finally, activity and sleep patterns were measured using the Drosophila Activity Monitor (DAM). It was found that metformin treatment significantly increased food intake and interaction in wild type flies, while also marginally disrupting normal sleep patterns. This result helps verify a direct connection between metformin treatment and the modification of cellular metabolism.

Lay Summary

Type 2 diabetes is characterized by the inability of the body to properly use insulin. When food is broken down into glucose (the primary sugar used by cells), it begins to accumulate in the blood, raising blood sugar. When this happens, the pancreas releases insulin, which acts as a signal to cells to take up the sugar and use it for energy. However, when blood sugar levels remain high for prolonged periods, tissues become less sensitive to insulin, and the sugar stays in the blood, resulting in serious health complications.

Metformin is a popular drug used to treat type 2 diabetes. Metformin primarily increases tissues' sensitivity to insulin, decreasing the amount of sugar in the blood without changing the amount of insulin being produced. Metformin is able to do this by activating cellular signals that govern metabolism. While metformin's anti-diabetic effects have been clinically proven, the signals activated by metformin lead to a vast array of effects that haven't been comprehensively studied.

This thesis uses fruit flies, a common and convenient model organism for genetics and molecular biology research, to identify metformin's effects on feeding and sleeping behaviors activated by the signals mentioned above. This was done by measuring the quantity of food eaten and minutes spent asleep each day. Here I found that metformin treatment increases food intake amount, body weight, and the number of "meals" eaten per day. I also found that metformin fragments sleep patterns, resulting in less efficient bouts of sleep.

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Introduction

According to the CDC (2023), more than 1 in 10 Americans have diabetes mellitus, and 90-95% of these suffer from non-insulin dependent, or type 2, diabetes. In the United States, diabetes is a top-10 leading cause of death (#8), as well as the number one cause of kidney failure, lower limb amputation, and adult blindness. Furthermore, since 2001, adult diabetes diagnoses have more than doubled. Type 2 diabetes is associated with many life-threatening health complications, such as nerve damage, eye damage, kidney disease (the #10 leading cause of death in the U.S.), and heart disease (the #1 leading cause of death in the U.S.) (Xu et al., 2022). Currently, there is no cure for diabetes, and the disease is mainly managed via the implementation of healthy lifestyle changes (CDC, 2023).

While there are no cures or substitutions for lifestyle changes, there are several drugs and therapies that have been developed to supplement healthy habits in the treatment of type 2 diabetes. Dimethylbiguanide, commonly known as metformin, has been the most prescribed oral treatment for type 2 diabetes for over 50 years (Hotta, 2019). Metformin is a very attractive treatment for diabetic patients due to its effectiveness and limited, mild side effects. Metformin increases the sensitivity of somatic tissues to insulin, allowing the body to properly utilize sugar in the blood. This effectively lowers blood sugar without affecting the amount of insulin being released by the pancreas.

Metformin exerts its anti-diabetic effects at the molecular level. The primary molecular targets of metformin include complex 1 of the electron transport chain (ETC), mammalian target of rapamycin complex 1 (mTORC1), and AMP-activated protein kinase (AMPK) (Vancura et al.,

2018). These proteins, especially mTORC1 and AMPK, regulate major interconnected signaling pathways that govern a wide range of bodily functions. The AMPK pathway is activated during periods of cellular stress and low nutrient availability; AMPK acts as a nutrient sensor that can detect when adenosine monophosphate (AMP) levels are higher than adenosine triphosphate (ATP) levels (Agius et al., 2020). When ATP is hydrolyzed, AMP levels rise, and AMPK is activated. Metformin is thought to inhibit the ETC, preventing the phosphorylation of AMP and artificially replicating low-nutrient conditions. The downstream effects of AMPK result in increased metabolism of glucose and fatty acids to generate ATP. Activated AMPK in turn decreases activation of mTORC1 (Laplante & Sabatini, 2009). The mTORC1 complex is exceptionally important for regulating cell proliferation and survival and is also a major component of the insulin pathway, which is dysregulated in type 2 diabetes and cancer (Arcidiacono et al., 2012; Laplante & Sabatini, 2009). The mTORC1 complex is activated by growth factors, and activation results in increased protein and lipid synthesis. In low-nutrient conditions, AMPK activates and mTORC1 is inhibited. This causes the cell to stop synthesizing macromolecules and save its energy. Together, mTORC1 and AMPK maintain energy metabolism homeostasis, which in turn is connected to the regulation of sleep homeostasis (Chikahisa et al., 2009; Laposky et al., 2008).

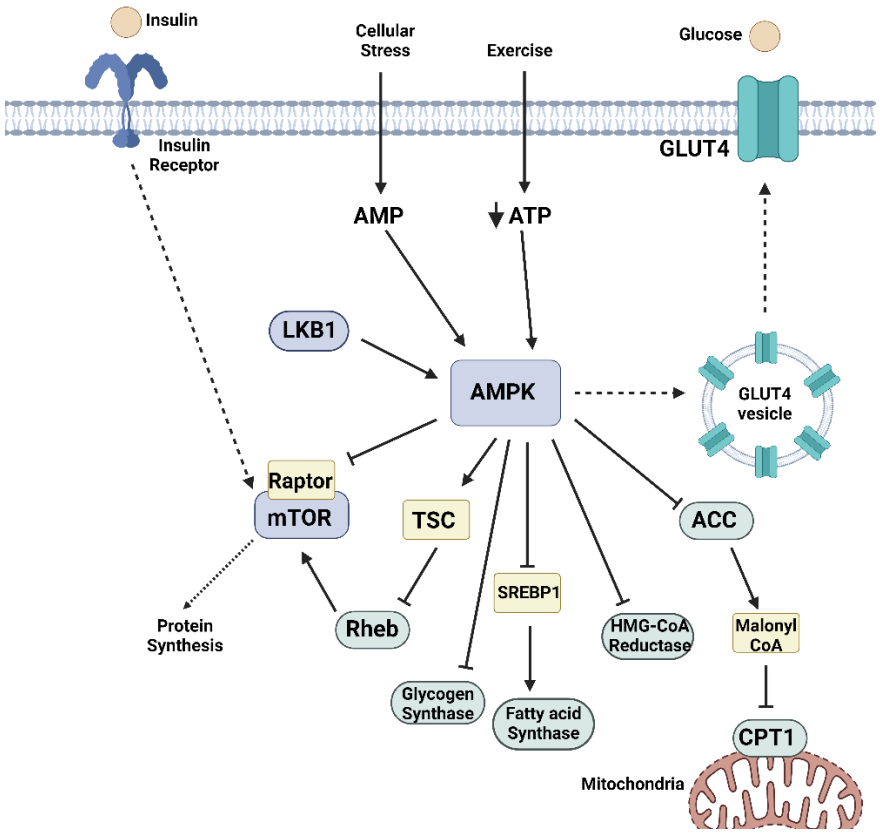

Figure 1. Simplified AMPK Signaling Cascade. Diagram of a simplified AMPK signaling pathway showing. AMPK is heavily involved in inhibiting anabolic pathways and triggering catabolic pathways. Created with BioRender.com.

Metformin's activation of these pathways is responsible for its desired hypoglycemic effects, but it may also lead to other important side effects. Research has shown that inhibiting the mTOR and insulin pathways leads to significant lifespan extension in roundworms, insects, and rodents. Also, mice and worms treated with metformin showed an increase in mean lifespan (Anisimov & Bartke, 2013; Johnson et al., 2013). The activation of AMPK and inhibition of mTOR are thought to help cells resist oxidative stress, thereby promoting longevity. Also, preliminary studies have shown that metformin treatment in human cancer patients led to the decreased expression of proliferation markers, potentially reducing tumor development

(Vancura et al., 2018). However, due to the interconnectedness of the AMPK pathway, it is extremely difficult to ascertain the precise mechanisms through which these side effects develop.

As the AMPK pathway regulates metabolism, which, in turn, regulates conditions like diabetes, oxidative stress, and even cancer tumorigenesis, it is important to study the effects of metformin on energy homeostasis to begin to ascertain the mechanisms governing the beneficial side effects described above. Since it is thought that *Drosophila melanogaster* do not engage in hedonic feeding, or pleasure feeding (Melcher et al., 2007), fruit flies serve as a convenient model organism for measuring differential feeding and sleep behaviors caused by metformin.

This thesis aims to address the question of how metformin affects feeding and sleep behaviors in fruit flies. Metformin treatment likely activates AMPK, and therefore also activates the fruit flies' low-nutrient response. This should cause the flies to consume more food, eat more meals, and gain weight. Furthermore, the flies may also exhibit different sleep patterns as a result of AMPK's regulation of energy homeostasis. To test these hypotheses, first, a feeding quantification assay was performed in wild type flies. This was done following a modified procedure for the Con-Ex assay (Shell et al., 2018). Feeding behavior and the number of "meals" consumed per day were measured using the Fly-to-Liquid-food Interaction Counter (FLIC). Starvation resistance and body weight assays were also performed to further analyze how metabolism may be modified by metformin treatment. Finally, sleep parameters were measured using the Drosophila Activity Monitor (DAM).

Food consumption was also measured in flies that were genetically modified using the UAS-GAL4 system. The UAS-GAL4 system is an effective method used to alter the expression of one or more genes in the offspring derived from the crossing of a driver and responder line (Brand & Perrimon, 1993). The responder line contains the gene of interest with an upstream activation sequence (UAS) that regulates transcription. The driver line contains a gene that codes for the transcription factor GAL4, which can bind and activate UAS sequences. The gene encoding GAL4 also has a tissue-specific promoter region. This allows different lines to express GAL4, and likewise the gene of interest, in specific tissues. When the driver and responder lines are crossed, the offspring express the gene of interest in the tissue of interest. The flies used to generate the hybrids in this study were all obtained from the University of Indiana Bloomington Drosophila Stock Center and are listed in Table 1; The phenotypes of the cross progeny are listed in Table 2. If metformin affects *Drosophila* metabolism by artificially activating AMPK, then modified flies with increased expression of proteins in the AMPK pathway should consume the same amount of food regardless of treatment.

Materials & Methods

Fly Stocks and Husbandry

Table 1. Fly Strains Used in Experiments

Stock Number	Name	Purpose	Source
5905	Iso31	Wild-type	Bloomington Drosophila Stock Center
4414	Act5C-GAL4	Ubiquitous Driver	
458	Elav-GAL4	Neuronal Driver	
36303	P{CaryP}attP2	attP2 RNAi control	

25931	UAS-AMPKalpha-RNAi	UAS-controlled RNAi knockdown of AMPK	
32110	UAS-AMPKalpha	UAS-controlled expression of constitutively active AMPK ($AMPK^{CA)}$	
8252	UAS-InR	UAS-controlled expression of dominant negative InR	

Mated wild-type (Iso31) male flies were used for most of the experiments. The UAS-GAL4 system was used to generate genetic hybrid strains. Non-wild type flies were collected and separated by sex within 8 hours of eclosion, before reaching sexual maturity. This ensured that flies were not mated. Then, driver line flies (lines 4414 and 458 in Table 1) were placed in the same vial with responder line flies (lines 36303, 25931, 32110, and 8252 in Table 1) and allowed to mate for 48 hours before removal. The offspring of each cross were collected and separated as previously described. For crosses with a balancer chromosome (see Table 2), flies presenting the phenotype associated with the balancer were removed and not used for experiments.

Table 2. Genetic Hybrids Generated for Feeding Quantification

Stock #'s Crossed	Genotype	Phenotype	Balancer?
4414 > 36303	Act-GAL4 > +	Crossing control	Y
4414 > 25931	Act-GAL4 > UAS-AMPK-RNAi	Ubiquitous knockdown of AMPK	Y

4414 > 32110	Act-GAL4 > UAS-AMPK^{CA}	Ubiquitous expression of AMPK^{CA}	Y
4414 > 8252	Act-GAL4 > UAS-(DN)InR	Ubiquitous expression of double negative InR	Y
458 > 36303	Elav-GAL4 > +	Crossing control	N
458 > 25931	Elav-GAL4 > UAS-AMPK-RNAi	Neuronal knockdown of AMPK	N
458 > 32110	Elav-GAL4 > UAS-AMPK^{CA}	Neuronal expression of AMPK^{CA}	Y
458 > 8252	Elav-GAL4 > UAS-(DN)InR	Neuronal expression of double negative InR	N

For all experiments, flies were reared and expanded in vials (23 mm x 95 mm) containing a standard solid media (Genesee Scientific, Cat#: 66-113). Flies were kept at 25 °C and 40-60% humidity on a 12-hr (9 AM – 9 PM) diurnal light cycle. For each experiment, newly emerged flies were collected in 72-hour cohorts and then allowed to mate for 24 hours before being separated by sex and transferred to experimental media.

For all food quantification and sleep monitoring experiments, the experimental media consisted of a fixed yeast concentration of 10% *w/v* (denoted from this point on as 10Y) and varying concentrations of sugar and metformin. The varying concentrations of metformin were used to test for a dosage-dependent effect on feeding behavior. Varying levels of sugar were also used because lower sugar concentrations may naturally activate the AMPK-mediated low nutrient response. This solid experimental media was prepared by boiling sucrose, brewer's yeast, and agar in water before adding preservatives (1% *w/v* propionic acid and 0.3% *w/v* nipagin dissolved in ethanol) and metformin.

For experiments utilizing the Fly-to-Liquid-food Interaction Counter (FLIC), liquid media was prepared by dissolving sucrose and 50 mg/L $MgCl_2$ (for consistent taste between batches) in DI water and adding metformin to treatment groups.

Finally, the solid media used for the starvation assays was prepared by boiling 1.5 % *w/v* agar in water, hand-pouring the solution into vials, and allowing the media to solidify.

Modified Con-Ex Food Consumption Measurement

The standard procedure for quantifying food intake used a modified version of the Con-Ex assay (Shell et al., 2018). Experimental media was mixed with 1% *w/v* FD&C Blue No. 1 dye and poured into caps. Flies were allowed to adjust to non-dyed experimental media for 5 days in before being transferred to empty vials topped with the prepared caps. The flies were kept on the dyed media for 48 hours before being removed. After removal, 3.5 mL of DI water were added to each vial and was vortexed. From each vial, 3 replicates of 200 µL each were loaded onto a 96-well plate. The optical density (OD) of each sample was then measured at 630 nm in a spectrometer.

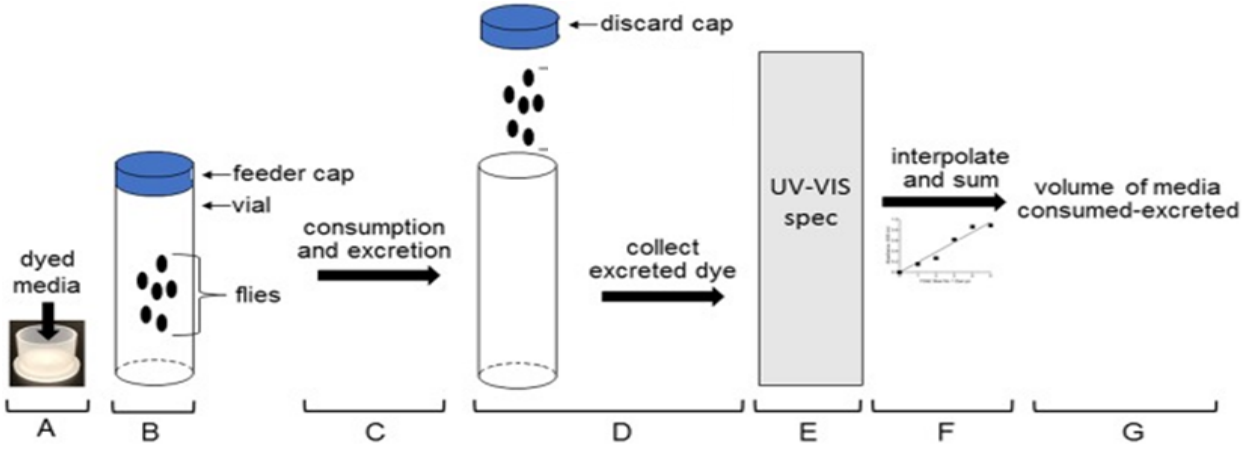

Figure 1. Diagram of Modified Con-Ex Procedure. Schematic of the steps for the Con-Ex method of *Drosophila* food quantification. Adapted from *Figure 1* (Shell et al., 2018).

Feeding Measurement at Variable Metformin and Sugar Concentrations

First, mated male Iso31 flies were maintained on 30 different experimental diets: media was prepared with 6 different sugar concentrations (0%, 1%, 2.5%, 5%, 10%, 20% *w/v*) and 5 different metformin concentrations (0, 2.5, 5, 10, 20 mM). After a 5-day entrainment period, flies were transferred to blue cap vials for 48 hours before removal. The OD was then measured for each sample as described above. For each sugar-metformin combination, 4-5 vials with 3 flies each were used.

Dose-Dependency Confirmation

After the sugar-metformin combination with the most robust results was identified, mated male Iso31 flies were entrained on 10SY media for 5 days before being placed into capped vials for 48 hours. The treatment group was placed on media with 10 mM metformin added, and the control group was placed on media with an equivalent amount of DI water added. After 48 hours on the cap food, the flies were removed, and the OD was measured. For each condition, 10 vials of five flies each were used.

Feeding Measurement in Genetically Modified Flies

Offspring of the crosses detailed in Table 2 were also entrained on 10SY media with 10 mM metformin supplied to treatment groups for 5 days before being transferred to capped vials for 48 hours. The OD was measured using the procedure outlined above. For each condition, 7-10 vials of 3-5 flies each were used.

Food Interaction Measurement

Feeding interactions were measured in wild type flies using the Fly-to-Liquid-food Interaction Counter (FLIC) developed by Sable Systems (Ro et al., 2014). This device runs an electric current through liquid media and records the increase in resistance that occurs when a fly contacts the media. This allows for the precise measurement of how often flies taste and consume food throughout a period of time.

Mated male Iso31 flies were loaded without anesthesia into individual arenas on the FLIC device (Figure 2) designed by Sable Systems (Ro et al., 2014). A food reservoir containing liquid media with 20% *w/v* sucrose and 5 mM metformin was connected to six arenas on one side of the Drosophila Feeding Monitor (DFM), while another food reservoir containing 20S liquid media without metformin was connected to the other side. The FLIC was run for 48 hours before termination.

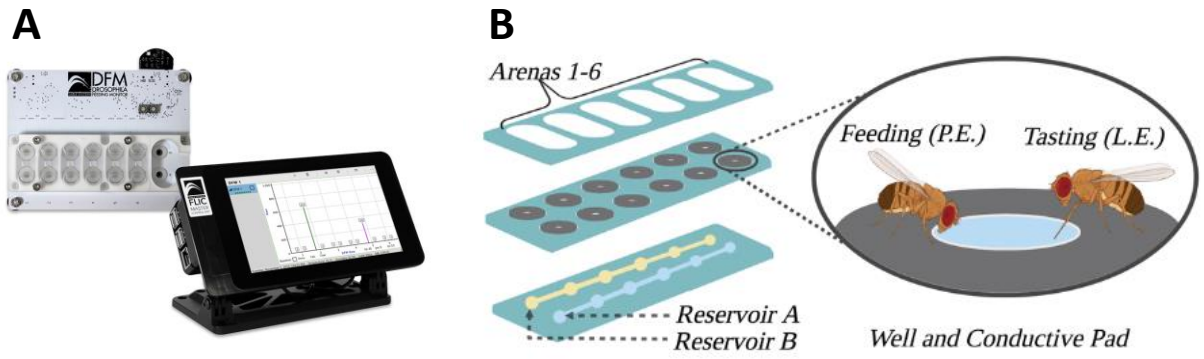

Figure 2. Images of FLIC Apparatus. (A) Photograph of FLIC Master Control Unit (MCU) and Drosophila Feeding Monitor (DFM) (Sable Systems, 2023) (B**)** Diagram showing the 12 arenas on the DFM, as well as the individual reservoirs associated with each row. Diagram also shows the difference between feeding events (proboscis extension) and tasting events (leg extension) (Philyaw et al., 2022).

Sleep Measurement

Fly activity levels and sleep rhythms were measured using the DAM system with standard established protocols (Trikinetics, n.d.; Pfeiffenberger et al., 2010). Solid media from the variable concentration experiment was added to small glass tubes. Following the dosage screening experiment, the flies were individually anesthetized with CO_2 and introduced to a glass tube with the same media the flies had been on for the previous experiment. About 15 flies per diet were loaded. The tubes were then loaded onto the DAM boards (Figure 3). The DAM boards are designed to shine an infrared laser through the center of the glass tubes. The device then recorded each time the laser's path was interrupted by the movement of the fly in the tube. A high concentration of disturbances per unit time indicates hyperactivity, while long periods of no disturbances indicate sleep bouts. The flies remained in the tubes for 2 days before the experiment was terminated. When analyzing the data, the first 24 hours were censored to account for CO_2 shock.

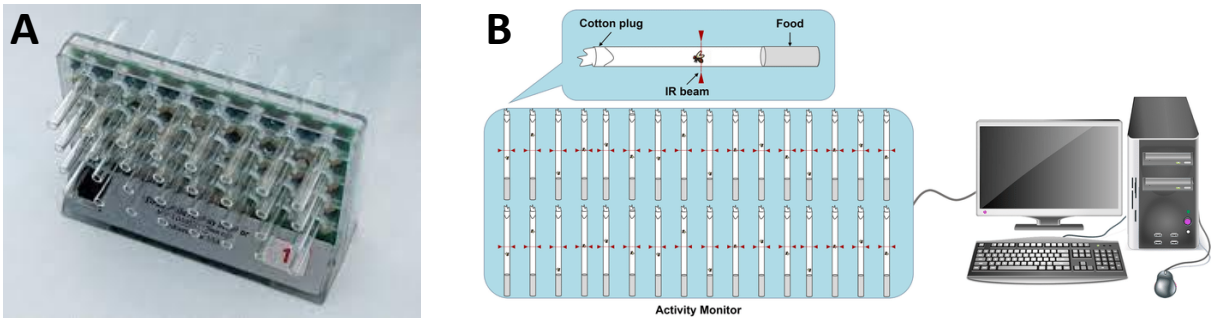

Figure 3. Images of Assembled DAM System. (A) Photograph of Assembled DAM board (Trikinetics, n.d.). (B) Diagram of DAM tubes with beam placement (Karam et al., 2022).

Body Weight and Starvation Assays

Following the large-scale feeding confirmation experiment, the wild type flies were removed from the cap vials and anesthetized with CO_2. The flies were randomized and separated into groups of five. An empty vial was tared on the scale, and groups of five flies were added to the vial and their masses were recorded.

After being weighed, the flies were transferred to vials of starvation media prepared as described above. The genetic hybrid flies were also randomized and transferred to starvation media following the modified Con-Ex assay. After being transferred to the starvation media, the number of dead flies in the vial was counted and recorded every 2-8 hours until all the flies were observed dead.

Statistical Analysis

A two-way ANOVA following the Tukey HSD post-hoc test was used to assess the impact of sugar and metformin concentrations on food consumption, sleep, and feeding behaviors. A student's t-test was used to compare the mean of food consumption and body weight between the control and 10 mM metformin. A log-rank test was used to compare survival curves for starvation resistance using OASIS2 (https://sbi.postech.ac.kr/oasis2/) and JMP 14 (SAS Inc.)

Results

Metformin Significantly Increases Feeding, Body Weight, and Starvation Resistance in Wild Type Flies

Metformin is thought to work by activating the AMPK nutrient sensor, causing a low-nutrient response that reduces anabolic activity and increases catabolic activity (Agius et al., 2020; Laplante & Sabatini, 2009; Vancura et al., 2018). Therefore, treatment with metformin should increase food intake and interaction. To test this hypothesis, food intake was quantified using the modified Con-Ex Assay detailed above.

Metformin treatment was shown to significantly increase the volume of food consumed compared to control groups ($p < 0.01$ two-way ANOVA). While food intake did increase in flies given metformin compared to control groups, there is only a suggestion of a dose-dependent trend. At 10SY, there is a robust increase in feeding between control and treatment groups ($p < 0.05$, two-way ANOVA), and there seems to be a dose-dependent trend in all dosages except 10 mM. This condition was selected for further feeding quantification experiments to determine if food intake significantly increases with 10 mM of metformin at 10SY, and potentially establish that there is a dose-dependent trend. There is also a mild trend that metformin's effect on food intake is stronger at higher sugar concentrations ($p = 0.08$, $F(20,111) = 1.55$). From this experiment, the conditions showing the most robust increase in food intake when compared to relative control groups were 10% sucrose ($p < 0.05$ two-way ANOVA; this condition was used for

all further feeding experiments with the medium metformin concentration [10 mM]) and 20% sucrose with 5 mM metformin (p = 0.0248; this condition was used for the FLIC experiment).

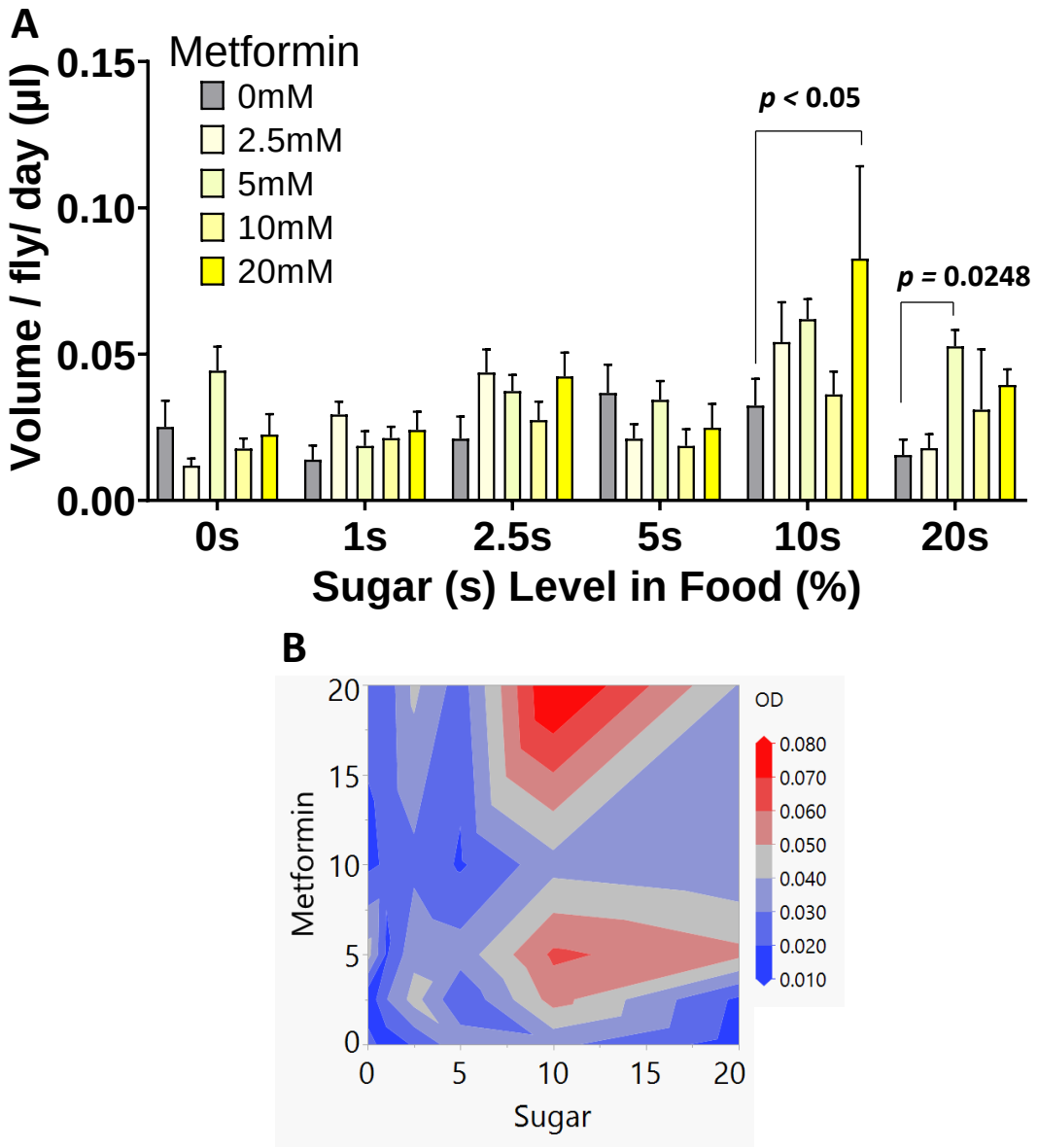

Figure 4. Daily Food Consumption of Individual Wild Type Flies on Variable Sugar Media. (A) Volume of food consumed per fly per day across different concentrations of sucrose and metformin. Data are expressed as the mean ± SEM. Two-way ANOVA, (for each condition, ~5 replicates were used with ~3 flies per replicate). (B) Heatmap illustrating relative measured OD across conditions. Highest OD was observed at sugar concentrations ≥ 10% and metformin concentrations ≥ 5 mM.

Metformin continues to significantly increase food consumption in wild type flies when retested on 10SY media (Figure 5, p = 0.0249). The mean volume of food consumed by each fly each day was approximately 31% higher in flies treated with 10 mM of metformin compared to flies on control media.

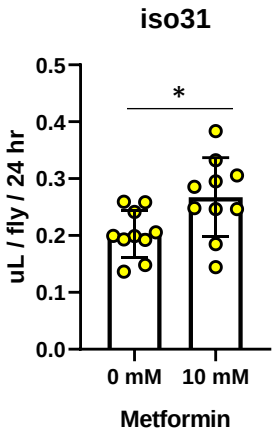

Figure 5. Daily Food Consumption of Individual Wild Type Flies on 10SY Media. Volume of food consumed by individual flies per 24 hours. Data are expressed as the mean ± SEM. * p ≤ 0.05 (p = 0.0249), unpaired t-test with Welch's correction, n ~10 replicates with ~5 flies per replicate, totaling ~50 flies per condition.

To test if metformin treatment caused a difference in feeding behavior, the FLIC was used to measure the number of feeding interactions performed by individual flies over time. Metformin treatment not only led to an increase in the quantity of food consumed, but also an increase in fly-food interactions in wild-type flies. In the group treated with metformin, an increase in the mean number of licks (Figure 6B, $p < 0.05$, two-way ANOVA) and feeding events (Figure 6A, $p < 0.0001$, two-way ANOVA). A "lick" is defined as a feeding interaction where the signal exceeds a calculated threshold above the baseline (Ro et al., 2014). These feeding interactions are distinct from tasting interactions, i.e. licks represent when food is being

consumed rather than just being tasted. A feeding event is defined as a series of contiguous licks. Feeding events are analogous to meals, while licks are analogous to bites of the meal.

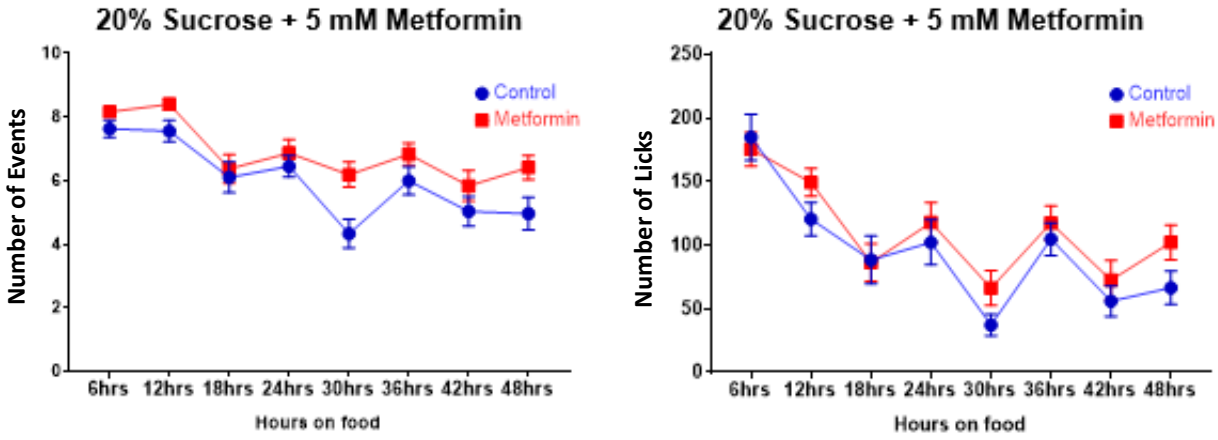

Figure 6. Feeding Interactions of Individual Wild Type Flies on 20S10Y Liquid Media. (A) The number of feeding events occurring within 6-hour bins measured from when flies were introduced to wells. (B) The number of licks occurring within 6-hour bins measured from when flies were introduced to wells. Data are expressed as the mean ± SEM. Two-way ANOVA, n = 12 flies per condition.

As hypothesized, wild type flies treated with metformin showed an increase in both interactions with food and quantity of food consumed. Since metformin significantly increases the amount of food consumed by wild-type flies, it likely affects metabolism. Therefore, the body mass of flies given metformin should be increased when compared to control flies.

To test this hypothesis, flies were weighed after being treated with metformin. After 7 days of treatment, flies in the treatment group showed a significant increase in body weight when compared to control flies (Figure 7, $p = 0.0177$).

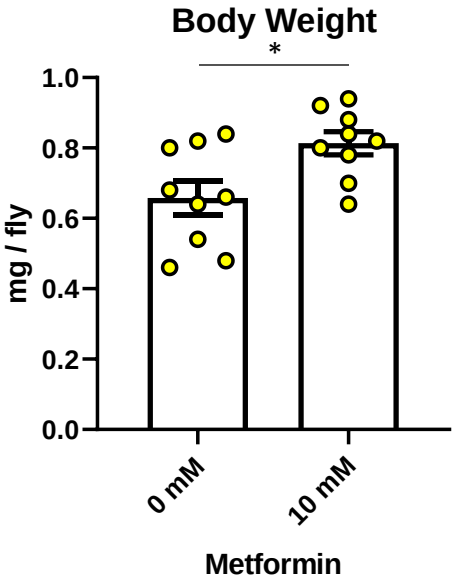

Figure 7. Body Weight Measurement of Wild Type Flies. End-point body mass measurements of individual flies measured in mg following 7 days on experimental media. Data are expressed as the mean ± SEM. * $p \leq 0.05$ ($p = 0.0177$), unpaired t-test with Welch's correction, n = 9 replicates of 5 flies each per condition.

Finally, as metformin is shown to significantly affect the metabolism of wild-type fruit flies, metformin treatment likely has an effect on individual flies' starvation resistance. Increased feeding and metabolic rates would likely indicate that flies treated with metformin would have a lower starvation resistance than flies given a control diet (Brown et al, 2019).

To test this hypothesis, a starvation assay was performed after the flies underwent their respective treatments for 7 days. Indeed, wild-type flies treated with metformin showed a

significantly different starvation resistance, however, it was increased (Figure 8, p = 0.042, log-rank test) when compared to flies on a control diet.

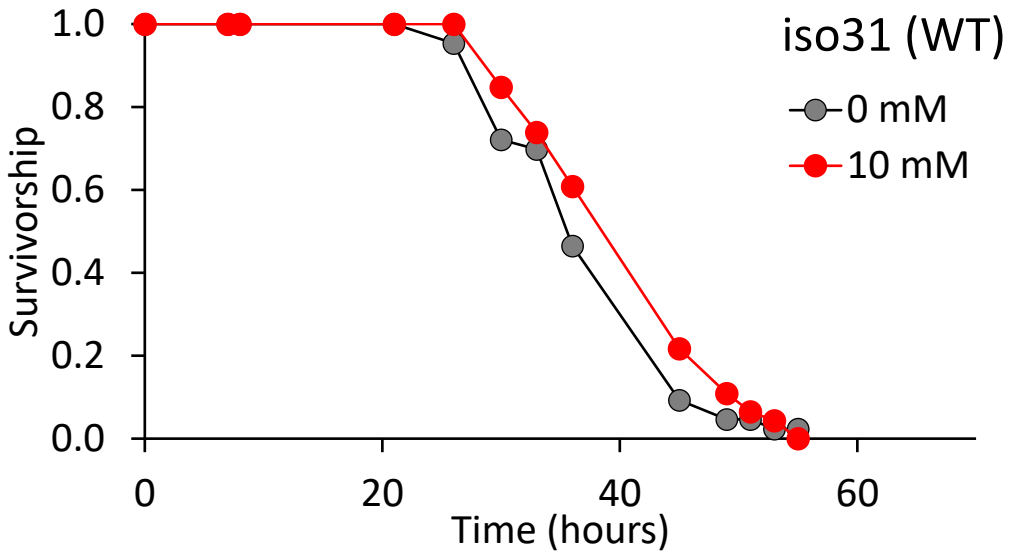

Figure 8. Starvation Survivorship of Wild Type Flies. Survivorship curve of flies on starvation media (1% *w/v* agar) following 7 days on experimental media. Data are expressed as a percentage of total flies still alive at the time of observation. P = 0.042 by log-rank test, n = 46 (0 mM); n = 47 (10 mM).

Flies Expressing Constitutively Active AMPK Did Not Show a Significant Increase in Food Intake

The AMP-activated protein kinase pathway (as well as the major pathways connected to it) is one of great physiological significance (Agius et al., 2020; Chikahisa et al., 2009; Steinberg & Kemp, 2009). Therefore, the modification of the genes associated with these pathways is risky. As a result, the majority of the crosses set up in Table 2, lead to a decrease in healthy progeny produced and an increase in lethality. These results are described in Table 3.

Table 3. Observed Developmental Limitations and Lethality

Cross Genotype	Phenotype	Observed Condition
4414 > 36303	Crossing control	No Issue
4414 > 25931	Ubiquitous knockdown of AMPK	Only flies with balancer phenotype emerged
4414 > 32110	Ubiquitous expression of $AMPK^{CA}$	Lower fecundity compared to both parental lines
4414 > 8252	Ubiquitous expression of double negative InR	Lethal
458 > 36303	Crossing control	Lower fecundity compared to parental lines
458 > 25931	Neuronal knockdown of AMPK	Lethal
458 > 32110	Neuronal expression of $AMPK^{CA}$	Lethal
458 > 8252	Neuronal expression of double negative InR	Lethal

Due to the limited number of healthy flies produced by the crosses, 4414 > 32110 (flies that ubiquitously expressed $AMPK^{CA}$) was the only line beside the control line (4414 > 36303) able to be utilized in experiments. However, while enough offspring were generated from the cross to run experiments, the flies expressing $AMPK^{CA}$ displayed a shorter lifespan than expected – about 50% of flies died within 7 days. This is much lower than the accepted average lifespan of wild type *Drosophila* at approximately 2-3 months (Sun et al., 2013).

Even with the limited numbers of genetic hybrid flies, the Con-Ex assay was still able to be performed after 5 days of entrainment on 10SY food. If metformin primarily modifies *Drosophila* food intake via the activation of AMPK, then flies expressing $AMPK^{CA}$ should consume the same quantity of food regardless of metformin treatment. As with the wild type trial, the treatment group was on media supplemented with 10 mM metformin, and the control group was on media supplemented with an equivalent amount of DI water added (in order to

account for any dilution caused by the addition of the metformin solution). For both the crossing control line and the AMPK^{CA} line, there was a non-significant difference in the mean quantity of food consumed by individual flies (Figure 9). However, flies expressing AMPK^{CA} consumed significantly less food when compared to the crossing control line ($p = 0.0002$ for 0 mM metformin, and $p < 0.0001$ for 10 mM metformin).

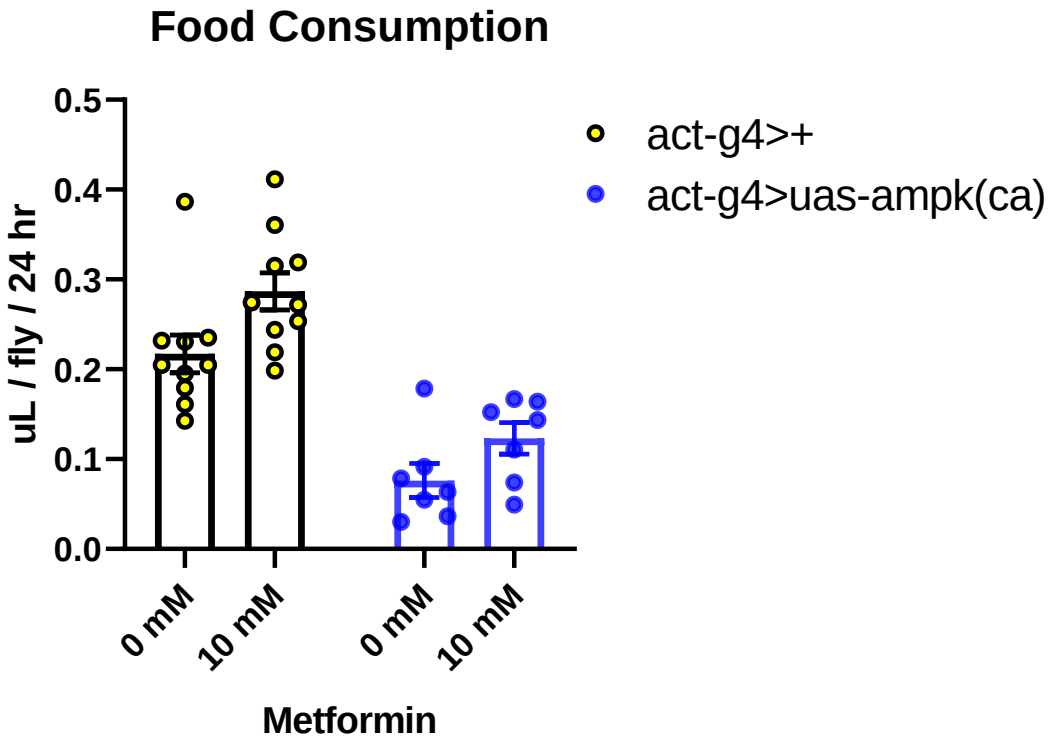

Figure 9. Daily Food Consumption of Individual Hybrid Flies on 10SY Media. Volume of food consumed by individual flies per 24 hours. Act-g4 > + represents 4414 > 36303; act-g4 > uas- AMPK^{CA} represents 4414 > 32110. Data are expressed as the mean ± SEM. P-values from two-way ANOVA, n = 10 replicates of ~5 flies each (4414 > 36303); n = 7 replicates of ~3 flies each (4414 > 32110).

Metformin Disrupts Normal Sleep Patterns in Wild Type Flies

AMPK acts as a nutrient sensor, triggering catabolic pathways whenever nutrient uptake is low (Agius et al., 2020), so it follows that metformin's activation of AMPK results in altered feeding behaviors in wild type flies. However, sleep also plays a major role in energy homeostasis (Chikahisa et al., 2009), and therefore metformin likely also affects sleep rhythms. If metformin promotes its metabolism-altering effects through AMPK, treatment likely increases sleep duration and quality as a means of maintaining energy homeostasis.

To test this hypothesis, sleep parameters were measured using the DAM. Following 7 days on the variable sugar media, flies were loaded into the DAM system and their activity patterns were recorded for 48 hours. Among the different concentrations of metformin (0, 2.5, 5, 10, and 20 mM), there were significant differences in sleep parameters (total sleep, total bouts asleep, and average bout length, $p < 0.001$, two-way ANOVA, Figure 10). There was no significant difference in sleep parameters between sucrose levels. Interestingly, it was observed that metformin simultaneously increased the total number of sleep bouts and decreased the length of the bouts ($p < 0.05$, two-way ANOVA), suggesting that metformin treatment may fragment sleep. This was especially evident in flies on 5S10Y media with 5 mM metformin (Figure 10C-E).

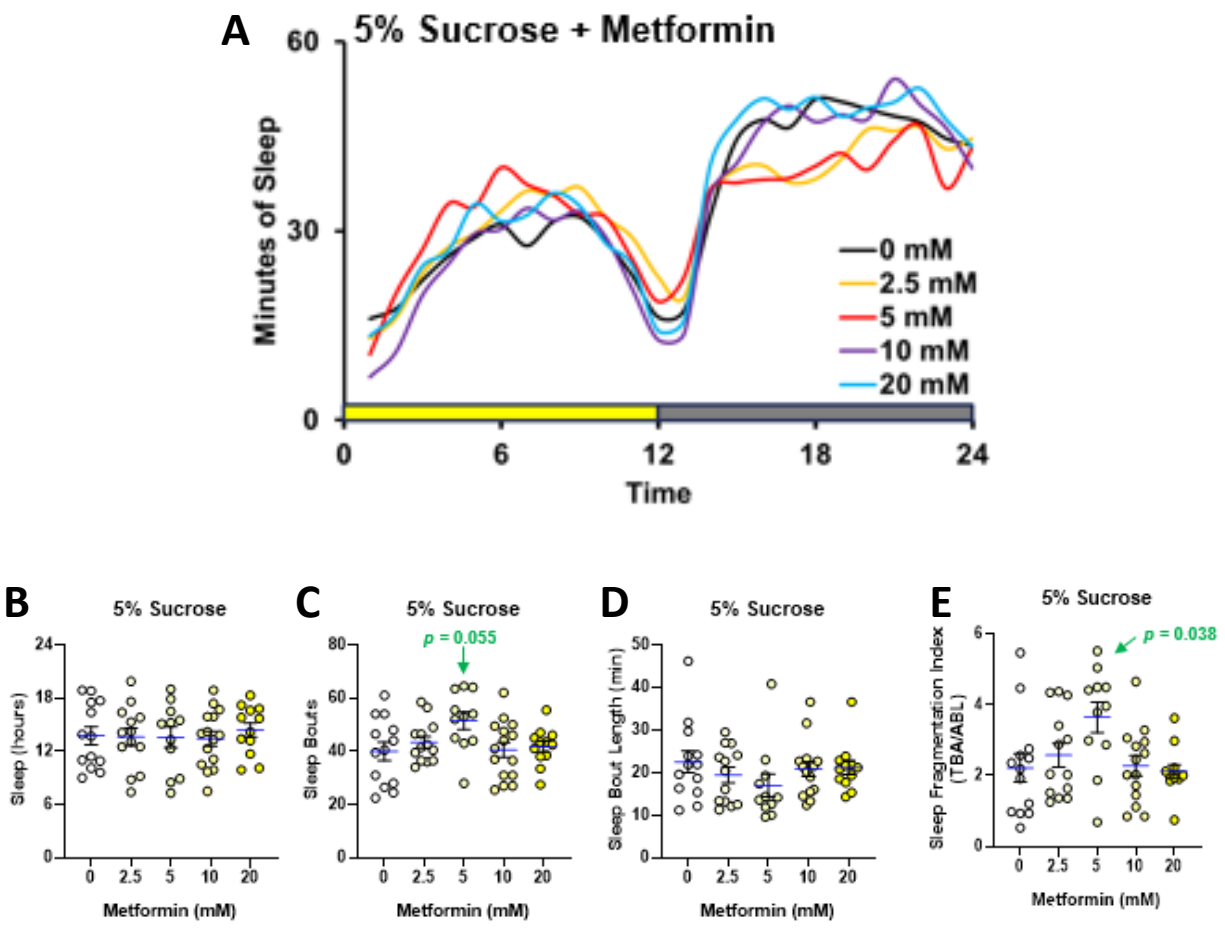

Figure 10. Mean Sleep Parameters of Flies on 5S10Y. (A) Average minutes of sleep over a 24-hour (12 hours light : 12 hours dark) period measured for each concentration of metformin on 5S10Y media. (B) Mean total time asleep in hours within a 24-hour period measured for flies on 5S10Y media. (C) Mean number of sleep bouts over a 24-hour period for flies on 5S10Y media. For 5 mM metformin, the difference in the number of sleep bouts is approaching significance ($p = 0.055$, two-way ANOVA). (D) Mean length of sleep bouts in minutes in the 24-hour period measured for flies on 5S10Y. (E) Sleep Fragmentation Index calculated as the total number of bouts (Figure B) divided by the average bout length (Figure C). A higher fragmentation index value indicates less continuous sleep. Flies treated with 5 mM metformin displayed significantly higher fragmentation compared to other groups ($p < 0.038$, two-way ANOVA). N = 12-15 flies per condition. Data are expressed as the mean ± SEM.

Discussion

Metformin is the most prescribed oral medication for type 2 diabetes in America, and has been for over 50 years (Hotta, 2019). The primary mechanism that metformin works through is the activation of AMP-activated protein kinase. This protein acts as a nutrient sensor, triggering pathways focused on the metabolism of macronutrients whenever cellular energy stores are depleted (Agius et al., 2020; Laposky et al., 2008; Vancura et al., 2018). It stands to reason that activation of the AMPK pathway would lead to changes in an organism's metabolism and feeding behaviors, especially in organisms that are thought to not engage in hedonic feeding, such as *Drosophila* (Melcher et al., 2007). This thesis aimed to identify the effects of metformin on feeding and sleeping behaviors in wild type flies. The confirmation of which genes may play a role in the mechanism of metformin treatment using genetically modified flies was another primary goal of this study.

For the quantification of feeding, the modified Con-Ex assay was chosen for several reasons. First, unlike radioisotope labeling, the Con-Ex assay allows food intake measurement without killing the flies (Deshpande et al., 2014). This allowed the measurement of body weight, starvation resistance, sleep, and feeding behavior following the food quantification experiments. Also, the Con-Ex method allows flies to feed in a natural way (compared to the CAFE assay) and can show reproducible differences between conditions (compared to PER). While radioisotope labeling and CAFE may produce more significant differences between conditions, the Con-Ex assay still shows sufficient differences, while also providing the benefits above.

This research shows that treatment of wild type, non-diabetic flies with metformin results in significantly increased food intake, food interaction, and weight gain (Figures 4-7). While this finding aligns with expectations, it provides valuable experimental evidence of a direct connection between metformin and feeding behavior modification. This result also indicates that metformin may indeed primarily work by artificially inducing a low-nutrient response. Furthermore, there may be a slight dose-dependent effect, where higher doses of metformin result in proportionally increased feeding behaviors. Finally, there is a mild trend that suggests that higher nutrient availability (i.e. higher sugar concentrations) results in an increased difference in food intake between control and treatment groups. This could be due to lower concentrations of sugar naturally activating a low-nutrient response, mitigating the effect of metformin.

Importantly, type 2 diabetes is primarily managed by implementing healthy lifestyle changes. Therefore, the governance of macronutrient intake is crucial for treating diabetic patients. If metformin, the most prescribed anti-diabetic medication in the U.S., drives increased food intake, then it could have counter-productive effects in some patients. Furthermore, most type 2 diabetic patients are overweight and/or obese, and both conditions are associated with an increased risk of developing heart disease (the top cause of death in the United States) (CDC, 2023; Xu et al., 2022). This study shows that metformin significantly increases body weight in fruit flies after one week of treatment. Identifying the underlying mechanisms through which metformin exerts these effects could help eliminate counter-productive side effects in at-risk patients with metformin prescriptions.

This study also shows that metformin increases starvation resistance in wild type flies (Figure 8). While the difference is only marginally significant ($p = 0.042$) the observed starvation resistance is likely still biologically significant. This is because the other metabolic data supports the result that flies treated with metformin underwent metabolic modification. This experiment will be repeated to confirm treatment group flies have an increased resistance to starvation. At first glance, this result appears to contradict expectations; animals with an increased metabolic rate (as seen in flies treated with metformin) would be expected to have lower starvation resistance (Brown et al, 2019). Furthermore, starvation (especially glucose starvation) results in the production of reactive oxygen species (ROS), which exert oxidative stress on the cell and eventually result in cell death. However, the activation of AMPK helps manage and prevent the formation of ROS during starvation conditions (Ren & Shen, 2019). Therefore, although metformin treatment results in increased metabolism, flies treated with metformin have an increased resistance to starvation, likely due to the increased activation of AMPK.

Oxidative stress is not exclusively caused by starvation; it is also a major mechanism of natural aging (Golden et al., 2002). Oxidative stress resulting from glucose starvation is also a result of cancer cells deregulating cellular metabolism (Vander Heiden et al., 2009). If metformin treatment activates AMPK and, in turn, reduces oxidative stress, this provides promising evidence that metformin could potentially be administered as an anti-aging or anti-cancer treatment in the future.

Currently, the effects of metformin treatment on sleep patterns are not well characterized. There is some evidence that metformin may cause sleep disorders such as insomnia in some human patients (Wiwanitkit & Wiwanitkit, 2012), and some reports indicate

that patients using metformin may also experience nightmares that disrupt sleep (Yanto et al., 2018). It is theorized that nocturnal hypoglycemia could lead to increased restlessness and vivid, abnormal dreams. However, there is also some conflicting evidence that indicates that metformin may actually increase total sleep time and efficiency (Kajbaf et al., 2014). This study shows that metformin treatment fragments sleep in non-diabetic, wild type fruit flies (Figure 10). Further research could potentially elucidate a concrete relationship between metformin treatment and the fragmentation or improvement of sleep patterns.

Finally, this thesis sought to quantify food consumption in flies with modified expression of different proteins in the AMPK pathway. Using the UAS-GAL4 system, flies were generated with modified expression of genes that would mimic the phenotype of metformin's proposed mechanism of action. Unfortunately, the ubiquitous and neuronal modification of these pathway proteins led to lethality and illness. In flies that ubiquitously expressed a constitutively active $AMPK^{CA}$ protein, the difference in mean food intake quantity between control and treatment groups was non-significant. This may indicate that metformin does indeed promote feeding via the activation of AMPK and its downstream pathways. However, flies expressing $AMPK^{CA}$ ate significantly less food compared to flies ubiquitously expressing the GAL4 protein with no driver line (Figure 9, $p = 0.0002$ between control groups, and $p < 0.0001$ between treatment groups). This could potentially indicate that there is another mechanism activated by metformin that regulates food intake. Considering the overall poor health of the generated flies over the course of the experiment, more extensive studies are needed to confirm this result.

Conclusion

This research shows that metformin treatment has significant effects on energy homeostasis regulation in wild type *Drosophila melanogaster.* Treatment with metformin was shown to increase mean daily food intake, mean body weight, number of meals per day, and starvation resistance. Also, flies treated with metformin displayed fragmented sleep patterns, showing an increase in the number of sleep bouts per 24 hours and a decrease in average bout length. These findings likely indicate that metformin exerts its effects (at least partially) by activating the AMP kinase pathway. Further research is needed to elucidate the cause of sleep fragmentation during metformin treatment; longer-term experiments utilizing both diabetic and non-diabetic flies should be performed. Finally, more genetically modified flies should be generated using a ubiquitous driver line that promotes less aggressive expression levels. As AMPK is known to be highly active in the mammalian liver and muscles (Agius et al., 2020; Kjobsted et al., 2018), crosses using drivers specific to tissues homologous to these should also be made. Finally, a gene-switch system that induces the expression of the gene of interest at a specific time could be used to trigger gene expression after development. This could help mitigate potential lethality associated with altering energy homeostasis of developing/larval flies.

Acknowledgments

This work was partially supported by the Summer Research Opportunity Program Award from the University of Louisville College of Arts & Sciences. I would like to profoundly thank my mentors, Dr. Dae-Sung Hwangbo and Mubaraq Opoola, who have worked tirelessly to support

me throughout my tenure as an undergraduate researcher at the University of Louisville. Thank you for always pushing me to be my best and inspiring me to continue to pursue a career in biological research. I would also like to thank the other members of the Hwangbo lab for their support, especially Breanna Beard, whose help with the DAM system was indispensable. I would like to thank my committee members Drs. Rafael Demarco and Nicholaus Noles for providing me with the opportunity to present the culmination of my research experience as an undergraduate. Finally, I would like to sincerely thank the University of Louisville Department of Biology and Honors Program for providing me with an excellent education that will serve me for the rest of my life.

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