Effect of Metformin on the Lifespan and Health Span of Drosophila

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I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration except for preliminary studies conducted by the labs of P. Darrell Neufer, Ph.D and Alexander Murashov, M.D., Ph.D. This work as not been submitted elsewhere as coursework for this or another degree.

Signed:  **Date:** 11/18/21

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Abstract:

Metformin has been the most used drug to treat diabetes for nearly 70 years. It reduces the pressure on the mitochondria from over calorific foods (i.e., fast foods), which termed the Western Diet. Metformin's ability to mildly decrease the efficiency of mitochondrial energy transformation has led to speculation that metformin may also promote healthy aging and extend longevity in non-diabetic, otherwise healthy individuals. Using the *Drosophila* model, we hypothesized that metformin, by decreasing mitochondrial and whole body bioenergetic efficiency, will increase lifespan in flies on the Western Diet but either decrease or have no effect in flies on a standard low-fat diet. Initial studies determined that in flight muscle mitochondria, titration of metformin (1-200 mM) or methyl-triphenylphosphonium (cation; mTPP; 0.01-0.25 mM) in vitro experiments induced a dose-dependent decrease in ADP-stimulated oxygen consumption rate (JO2; normalized to complex IV activity). For in vivo studies, flies were fasted for 20 hours and then provided food containing mTPP (0-1.0 mM) with blue dye for two or four hours. Surprisingly, despite evidence of food consumption, no differences in ADP-stimulated JO2 were detected, suggesting either the flies did not consume enough food or that mTPP was not absorbed or ineffective in vivo. Results from Western Diet Studies showed no change in mitochondrial efficiencies for *D. melanogaster* however, for *D. simulans*, there was a lower mitochondrial efficiency in flies on the Western Diet that also exercised.

Acknowledgments

I would sincerely like to thank the people who have supported me throughout this study: Dr. Alexander K. Murashov, Dr. Chien-Te (Peter) Lin, Bevan Lewellen, Kang Tung, Elena S. Pak, Dr. Cameron A. Schmidt, Katherine A. Buddo, Dr. Susan B. McRae, the Department of Biology, East Carolina University, and my parents, Stephen and Catherine Chan. I am especially grateful to my primary faculty mentor, Dr. P. Darrell Neufer, for his knowledge and guidance.

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Introduction:

Aging is an inevitable part of life that brings many physiological changes to the body from graying hair and wrinkles to aging-related diseases. Recent epidemiological studies have suggested that metformin, a drug commonly used to treat diabetes, also protects against several aging-related diseases in diabetic patients including cancer, neurodegenerative disease, and cardiovascular disease. These data have led to speculation that metformin may also promote healthy aging and extend longevity in non-diabetic otherwise healthy individuals. This research project is based on the premise that discovering more about metformin's molecular mechanism will provide a better understanding of the aging process and how aging-related diseases may be better treated.

Dubbed Glucophage or "glucose eater", metformin has been proven useful in treating type 2 diabetes due to its pleiotropic ability to decrease hepatic glucose production, decrease intestinal absorption of glucose, and increase the efficiency of sugar uptake in cells.[1] Although metformin has been commonly used for the past 70 years, its primary molecular mechanism of action remains elusive. Current research has shown that metformin impacts multiple pathways by accumulating in the mitochondria and inhibiting Complex I of the electron transport chain.[2, 3] However, it is unclear how metformin would be able to penetrate the inner membrane of the mitochondria as no specific transporter has been identified as of yet.[4] There is also little evidence that metformin accumulates to high enough concentrations in tissues to affect Complex 1 activity.[5] Even though *in vitro* studies have shown that a high concentration of metformin in the

mitochondria (IC_{50} ~20 mM)[6, 7] is needed to inhibit Complex 1, *in vivo* studies show that metformin accumulates in tissues to only low µM levels.[5, 8]

Within the mitochondria, the Electron Transport System (ETS) drives the generation of adenosine triphosphate (ATP) for the cell due to the difference in charge across the membrane. In the ETS, oxygen "pulls" on electrons from complex carbon molecules due to oxygen's very high reduction potential. As electrons are donated into the mitochondrial matrix from NADH in Complex I and $FADH_2$ in Complex II, Complexes I, III, and IV serve as proton pumps to create a proton gradient that in turn drives ATP production. Although Complex II does not directly pump protons, it serves as another source of electrons for Complexes III and IV that are also proton pumps. The pumping of protons to the outer surface of the membrane creates the proton motive force (ΔG_{pmf}) which regulates the ETS by imposing a back pressure on the proton pumps. The exchange of electrons and protons generates a membrane potential across the inner mitochondrial membrane which in turn establishes and holds the energy (ATP/ADP via ATP synthase) and redox ($NADP^+$/NADPH via nicotinamide nucleotide transhydrogenase; NNT) charges needed to support numerous cellular functions. Oxygen serves as the final electron acceptor and draws electrons from upstream catabolic pathways. This leads to the formation of water in an irreversible reaction that ultimately results in the formation of the cell's energy, ATP. The ETS is self-regulated through the free energy of ATP (ΔG_{ATP}) generated by the high ATP/ADP ratio which establishes a back pressure against the free energy of the ΔG_{pmf} driving the ATP synthase.

In addition to providing energy for the cell, mitochondria also generate, dispense, and remove numerous intracellular signaling effectors including hydrogen peroxide (H_2O_2).[9] Mitochondrial H_2O_2 (a reactive oxygen species) production and emission becomes elevated when there is a surplus of energy. This can be found in what is known as the Western Diet (WD). The WD is notorious for its high fat and sugar content and has been strongly linked to the increased prevalence of obesity, type 2 diabetes, cardiovascular diseases (CVD), inflammation, and behavioral disorders including bulimia and binge-eating.[10] High salt content is another considerable element in the WD that contributes to a direct increase of high blood pressure, CVD, stroke, and renal diseases.[11] An oversupply of energy (from the WD) puts pressure on the ETS and when not relieved, causes an increase in H_2O_2 production. Thus, elevated mitochondrial H_2O_2 levels and the consequent shift in the cellular redox environment to a more oxidized state have been linked to the etiology diet-induced insulin resistance[9] and other aging related diseases.[12] While elevated mitochondrial H_2O_2 levels have been shown to have detrimental effects on the body, there are several organic cations, including metformin, that decrease mitochondrial H_2O_2 production at Complex I. The formal positive charge carried by metformin and other cations allow them to accumulate in the negatively charged mitochondrial matrix, reducing the net electrochemical charge across the inner membrane. Preliminary force-flow data reveals that the presence of organic cations decreases the net free energy available (ΔG_{pmf}) to support ATP synthesis during low to moderate workloads. This translates *in vivo* to a slightly lower displacement from equilibrium for the ATP hydrolysis reaction (i.e., decreased [ATP]/[ADP] and thus, a lower Gibbs energy content). Since the Gibbs energy content of ATP hydrolysis reaction decreases exponentially as the displacement from equilibrium decreases, a higher rate of ATP utilization is required to support the reactions in ATP hydrolysis. Thus, when

there is a surplus of energy, metformin serves as a counterbalance as it makes the mitochondria less efficient which in turn relieves pressure on the ETS. This primary mechanism of action of metformin potentially explains why weight loss occurs in patients with metformin treatment and the discrepancy between intact cells and isolated mitochondria with respect to the concentration required to "inhibit" Complex I function.

Due to metformin's ability to lower the efficiency of the mitochondria, it has also been argued that metformin prolongs lifespan and health span (from aging-related diseases). When there is a high caloric intake, less efficient mitochondria have been shown to extend the median lifespan in *C. elegans* by 40% when raised on 50 mM metformin.[13] There have also been studies demonstrating the same effect in mice; however too high of a concentration of metformin can lead to a significant decrease in lifespan.[14] None of the aging studies so far have evaluated the impact of metformin on lifespan with energy balance as a covariable. This research aims to study this covariable through different diets to determine a more wholistic impact of metformin on health span and lifespan. The *Drosophila* model will be used because of how easy and cost efficient it is to maintain fruit flies. In addition, fruit flies also have a relatively short lifespan of only several weeks which is advantageous for multiple runs of experiments.

Preliminary studies have shown a reduction in mitochondrial efficiency (low JO_2 rate) in Western Diet flies and a slight increase in Western Diet and Exercise flies (high JO_2 rate) (Fig.1).[10]

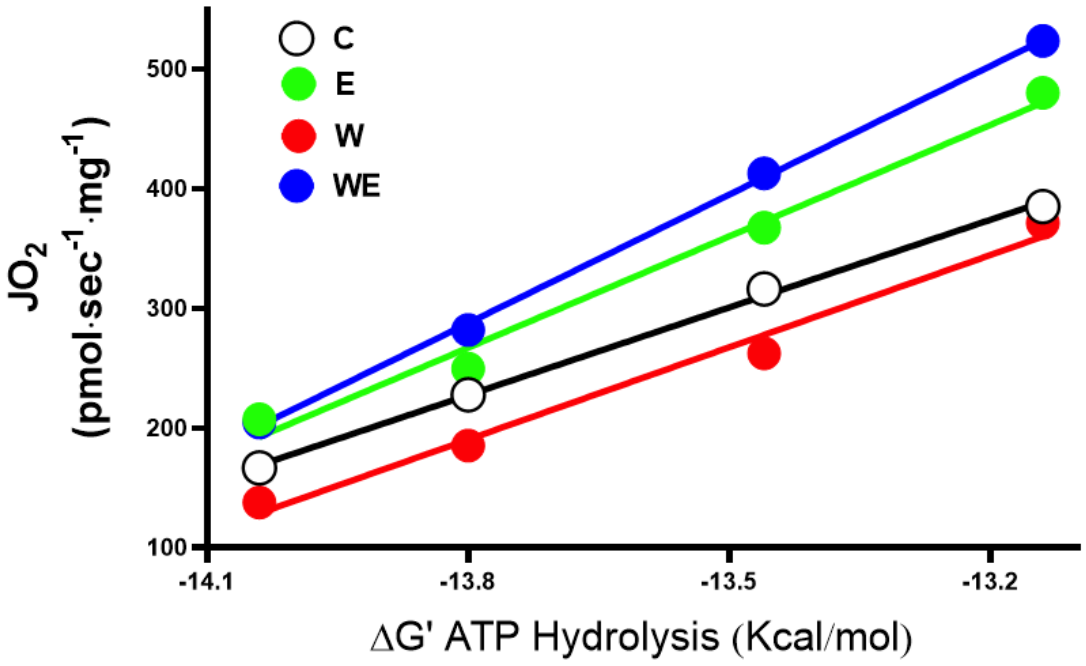


Figure 1. Data from preliminary study conducted by the labs of Dr. P. Darrell Neufer and Dr. Alexander Murashov.

This research will investigate whether metformin, similar to other positively charged molecules, works by inducing a mild decrease in the efficiency of mitochondrial energy transformation, a primary mechanism that could account for the protection against numerous aging-related diseases**.** Methyl-triphoniumphosphate (mTPP) was studied as it is a simpler cation typically used as a membrane marker to introduce cations into the mitochondria due to its ability to cross the lipid bilayer by non-carrier-mediated transport.[15] Preliminary *in vitro* experiments showed that it was more effective than metformin at lowering the % JO2, even at lower doses. The purpose of this study is to determine whether metformin or mTPP influences lifespan in the

Drosophila model, and to determine whether the influence of metformin depends on the dose, diet (i.e., high fat vs low-fat diet), and activity level (i.e., exercise).

This study attempts to determine if a mild reduction in mitochondrial bioenergetic efficiency though metformin extends lifespan. Due to metformin's ability to reduce the efficiency of the mitochondria, it is hypothesized that metformin will increase the lifespan in flies on a high-fat diet. It is also hypothesized that metformin will decrease or have no effect on flies on a standard low-fat diet because the metformin would release too much pressure on the ETS.

Methodology:

This study was a collaboration between Dr. Neufer and Dr. Alexander Murashov. The fruit flies were housed and cared for in Dr. Murashov's lab in The Brody School of Medicine and the data collection from the mitochondrial respiration experiments occurred in Dr. Neufer's lab in the East Carolina Diabetes Institute. The mitochondrial efficiency of *Drosophila melanogaster and Drosophila simulans* on several experimental combinations. *D. melanogaster* were commercially purchased and *D. simulans* used in experiments were derived from a colony established from six *D. simulans* isofemale lines collected in 2018 in Greenville, NC. Flies were identified according to the identification guide[16] and maintained as an outbred population. All flies were maintained on the standard Bloomington Formulation diet (Nutri-Fly® BF, Cat #: 66-112. Genesee Scientific Inc., San Diego, CA) in a climate-controlled environment at 24ºC under a 12-hour light-dark cycle and 70% humidity. Only male *Drosophila* were studied in the experiments as female flies respond poorly to exercise[17] and require extra care to clean their vials of eggs.

For mitochondrial respiration (JO2) experiments, multiple high-resolution oxygen consumption measurements were performed using the Oroboros Oxygraph-2K (Oroboros Instruments). The flies were sacrificed at the age of five days and at the same time each day. Flight muscles (cuticle) were isolated and examined as Dr. Neufer's lab has discovered that the flight muscle mitochondria in a single fly are very adept at using oxygen. All experiments were executed at 23ºC (room temperature, assumed fly body temperature at rest) in a 1 mL reaction volume with continuous stirring (500 rpm). To start, the flies were immobilized on a cold metal block (0ºC) and dissected by first removing the head followed by the wings, abdomen, and legs. The flight muscles were made accessible by carefully splitting the thorax cuticle. The thorax cuticle was then immediately transferred to a preweighed tube of the buffer for the experiment (different buffers for *in vitro* vs *in vivo*), weighed, and then the fly weight was calculated. The fly was then added to the respirometer chamber filled with 1 mL of the same respiration buffer.

In vitro Studies

Pilot *in vitro* studies were conducted to determine the effects of the metformin and mTPP on *Drosophila*. The buffer included Buffer Z (105 mM K-MES, 30 mM KCl, 10 mM KH2PO4, 5 mM MgCl2, 1 mM EGTA, 0.05% fatty acid-free bovine serum albumin, pH 7.1)[22] supplemented with 10 mM Creatine (Cr), 2 mM Malate, 5 mM Pyruvate, and 4 mM ADP. Metformin and mTPP were titrated in the final concentrations of 5, 25, 50, 75, 100, 150, and 200 mM and 0.005, 0.01, 0.025, 0.05, 0.075, 0.1, and 0.25 mM, respectively. These were compared to the control group where the drop in JO2 was monitored over the course of 30 minutes of flies in buffer with no added cation. mTPP was found to be more effective at lowering the mitochondrial respiration

at lower doses than metformin (Fig. 2). Thus, we decided to begin *in vivo* experiments with mTPP.

In vivo Studies

For *in vivo* experiments, flies were fasted for 20 hours and then treated with 0.075mM and 0.2mM mTPP in food for two hours. Blue dye was added to the food to indicate if the flies ingested the food. Flies were dissected, weighed in premeasured buffer (Buffer Z, 10 mM Creatine, 5 mM Pyruvate, 2 mM Malate, 1 U/mL HK, and 2.5 mM 2-DOG), and then added to the 1mL Oxygraph-2K chambers. ADP was titrated to a final concentration of 0.02, 0.05, and 2 mM. Results were expressed in two different ways: data normalized to tissue weight (from preweighing) and data normalized to TMPD Complex IV activating data (by adding 2 mM Ascorbate and 0.5 mM TMPD at the end of the mitochondrial respiration experiment). Results showed no difference between treatments even though there was evidence that the flies ingested the food. The same experiment was repeated twice, with treatment lasting four hours and seven days to test if two hours was not enough time for mTPP to incorporate into the fly cuticle. An additional 1 mM mTPP group was added to serve as a "megadose". These *in vivo* experiments were repeated with metformin by fasting the flies for 20 hours and then treating with 100 mM, 500 mM, and 1000 mM metformin for 24 hours and 7 days.

Western Diet Studies

Next, studies to determine the effects of the Western Diet (WD) on the *Drosophila* without the impact of Metformin or mTPP were conducted. These were based on previous experiments that studied male *Drosophila simulans.*[10] This preliminary data showed a lower mitochondrial

efficiency in flies on the WD and a higher mitochondrial efficiency in flies in the Western Diet and Exercise (WDE) group. This experiment was replicated with commercially available *Drosophila melanogaster* to determine if they would respond similarly to the diet and exercise. Using commercially available flies would also allow for future genetic studies due to the availability of gene knockouts. In addition to using *D. melanogaster* rather than *D. simulans*, data for the replicates was normalized to both Complex IV activating TMPD data and tissue weight. All flies, from the embryo stage, were raised on the standard Nutri-Fly Bloomington diet or Control Diet (CD) and after three to four days, the flies were transferred to their experimental conditions: Control Diet (CD), Western Diet (WD), and Western Diet and Exercise (WDE). A Western Diet and Fasting group (WDF) was added for the *D. simulans* experiments to offer another "treatment" for the Western Diet. These flies were on WD food for the first four days and then fasted for the fifth day. This fasting day served as another treatment for the WD as pressure on the mitochondria would be relieved from a decrease in nutrition. WD food was made based on the standard Nutri-Fly Bloomington diet with the following adjustments: 15% Nutiva USDA Certified Organic, non-GMO, Red Palm Oil, 15% Sucrose, 0.1 M NaCl; HFD-15%[15]. For flight exercise groups, vials containing flies will be housed in a 1-gallon clear plastic drum fishbowl (Petco, San Diego, CA) strapped to a horizontal platform attached to a motor. The motor was controlled by two timers initiating four motor revolutions spaced 14 seconds apart every five minutes. Each revolution causes the platform that the fishbowl rests on to rise and drop. This action drops the flies to the bottom of the vial which triggers flight. The exercise does not harm the flies was performed daily for seven hours for five days. The platforms on which the vials were on were dropped four times every five minutes for five hours a day. Once the flies drop to the bottom of the vial, they instinctively fly/climb up the vial, thus inducing exercise.

There has not been any evidence of harm to the flies from the induced exercise as they have enough time to rest between drops.

Mitochondrial respiratory control was assessed using the creatine kinase (CK) energetic clamp system (force-flow) as detailed in previously described studies[18, 19] with procedures modified to be carried out at 23ºC in a 1 mL reaction volume with continuous stirring. This is a relatively new approach developed by the Neufer lab that for the first time replicates the three free energies present *in vivo*, which is the only way to determine whether mitochondrial efficiency is different under one circumstance compared to another. Fly mitochondria was energized with 0.5 mM malate, 5 mM pyruvate, 5 mM proline, and 10 mM succinate. Phosphocreatine (PCr) was further titrated in the final concentrations of 3.75, 7, 13, and 20 mM corresponded to ΔGATP of −13.14, −13.46, −13.8, and −14.04 kcal/mol.

Results:

In vitro Studies

In vitro studies showed the control group JO2 decreased to 51% of the original JO2 by the end of the 30 minutes (Fig. 2). Lower JO_2 ratios suggest less efficient mitochondria while higher JO_2 ratios suggest more efficient mitochondria. The metformin group required 200 mM metformin to reduce the mitochondrial efficiency to 23% and the mTPP required a smaller dose of 0.25 mM mTPP to reduce the mitochondrial efficiency to 6%.

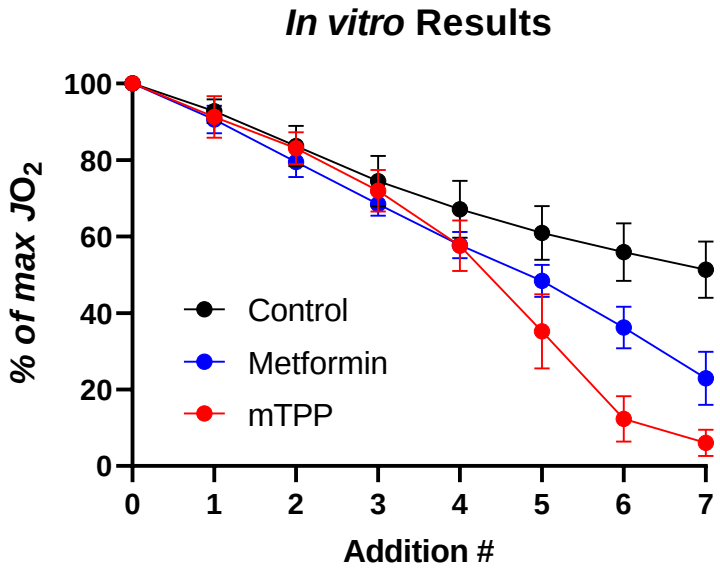


Figure 2. *In vitro* results.

In vivo Studies

After fasting *Drosophila* for 20 hours and treating with mTPP for two hours, no significant difference was found between mitochondrial respiration of 0.075mM, 0.2mM, and 1 mM mTPP. Treatment for four hours only showed a significant decrease of 0.022 ± 0.005 JO2 between the control and 1 mM mTPP basal groups normalized to Complex IV activating TMPD data (95% CI 0.01-0.04 [$p < 0.01$]) and a significant decrease of 0.014 ± 0.004 JO2 between the 0.075 mM mTPP and 1 mM mTPP basal groups normalized to Complex IV activating TMPD data (95% CI 0.001-0.03 [$p < 0.05$]) (Fig. 3).

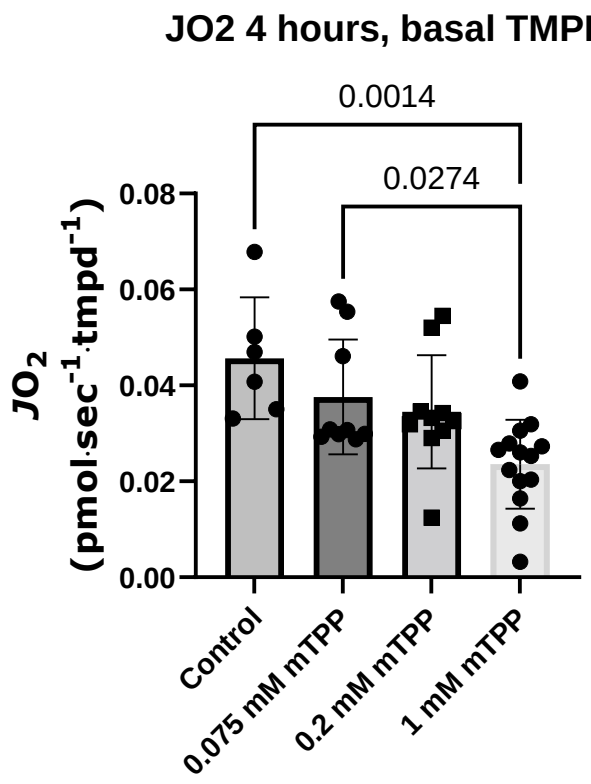


Figure 3. *In vivo* results from four hours on mTPP at basal rate normalized to TMPD data.

Treatment for seven days resulted in a significant decrease of 0.006 ± 0.001 JO2 between the control and the 0.075 mM mTPP basal groups normalized to TMPD (95% CI 0.003-0.009 [$p < 0.0001$]) (Fig. 4A). There is also a significant reduction of JO2 in the 0.05 mM ADP addition data normalized to Complex IV activating TMPD data. There is a significant decrease of 0.030 ± 0.008 JO2 between the control and the 0.075 mM mTPP groups (95% CI 0.01-0.05 [$p < 0.001$]), a decrease of 0.026 ± 0.007 JO2 between the control and 0.2 mM mTPP groups (95% CI 0.01-0.04 [$p < 0.005$]), and a decrease of 0.028 ± 0.008 JO2 between the control and the 1 mM mTPP groups (95% CI 0.01-0.04 [$p < 0.005$]) (Fig. 4B).

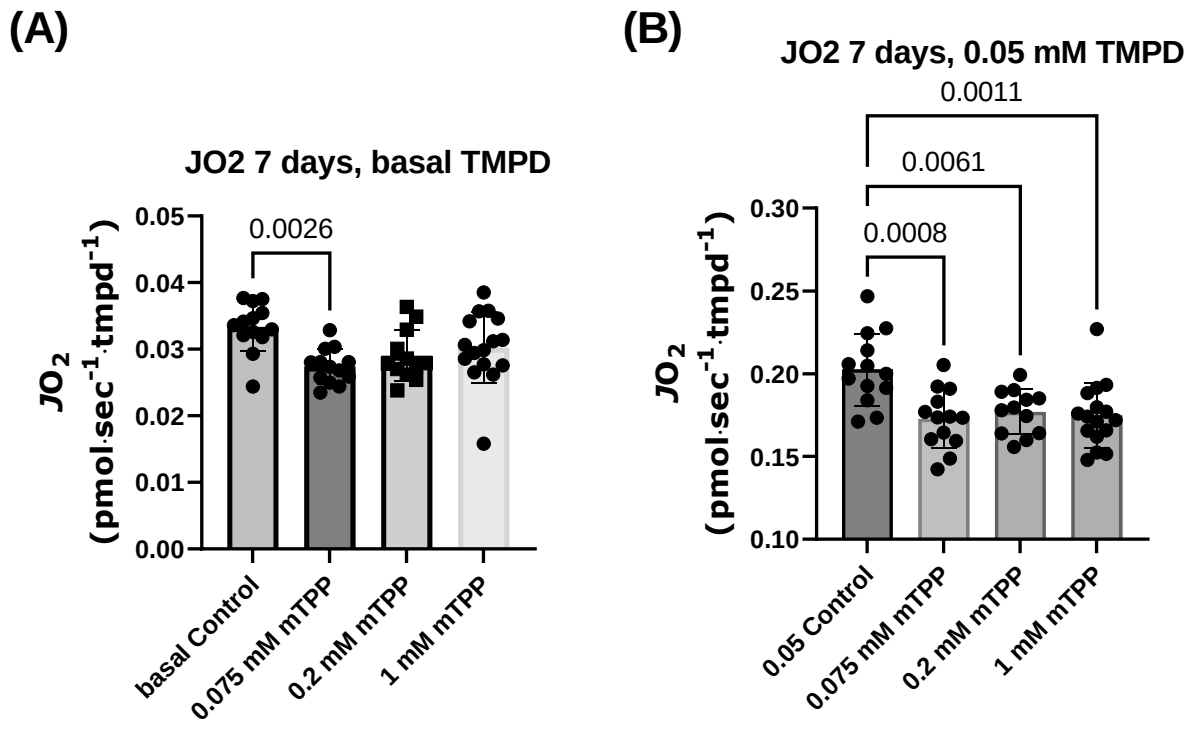


Figure 4. *In vivo* results from seven days on mTPP. (A): Basal rate normalized to TMPD data. (B): 0.05 mM ADP rate normalized to TMPD data.

In vivo experiments with metformin treatment of 24 hours showed a decrease of 100 ± 40 JO2 between the control and 500 mM metformin groups normalized to weight (95% CI 23.3-178.5 [$p < 0.05$]) and a decrease of 90 ± 40 JO2 between the control group and the 1000 mM metformin weight group (95% CI 6.03-179 [$p < 0.05$]) (Fig. 5A). After 0.05 mM ADP was added, there was a significant decrease of 170 ± 50 JO2 between the control and 500 mM metformin groups normalized to weight data (95% CI 54.7-176.5 [$p < 0.01$]) (Fig. 5B). There was also a decrease of 0.06 ± 0.02 JO2 between the 100 mM and 1000 mM metformin groups normalized to Complex IV activating TMPD data (95% CI 0.01-0.10 [$p < 0.05$]) (Fig. 5C).

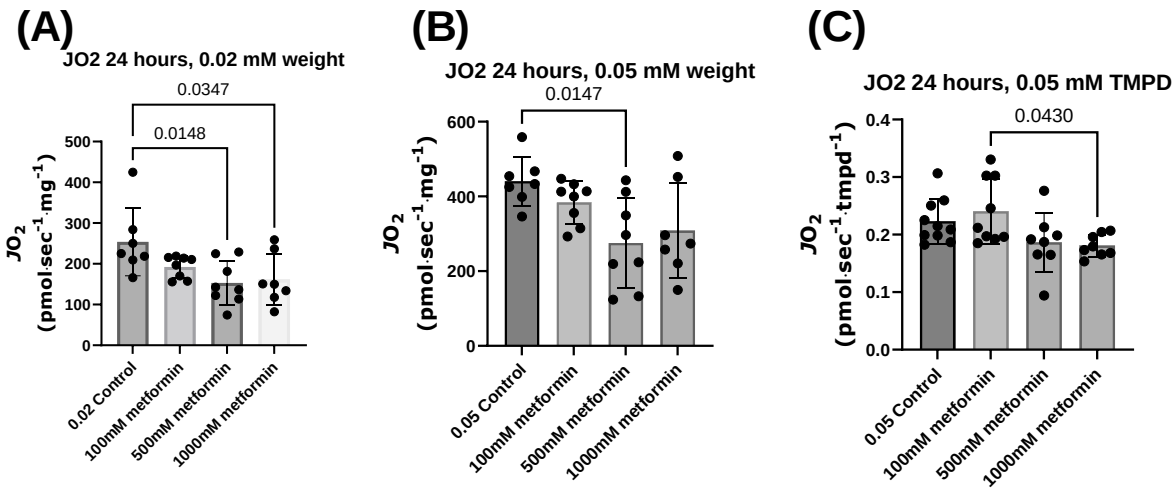


Figure 5. *In vivo* results from 24 hours on metformin. (A): 0.02 mM ADP rate normalized to weight. (B): 0.05 mM ADP rate normalized to weight. (C): 0.05 mM ADP rate normalized to TMPD data.

After treating for seven days, only the addition of 2 mM ADP showed any significant difference between the control group and the 100 mM metformin group. There was a decrease of 0.02 ± 0.01 JO2 (95% CI 0.002-0.043 [$p < 0.05$]) (Fig. 6).

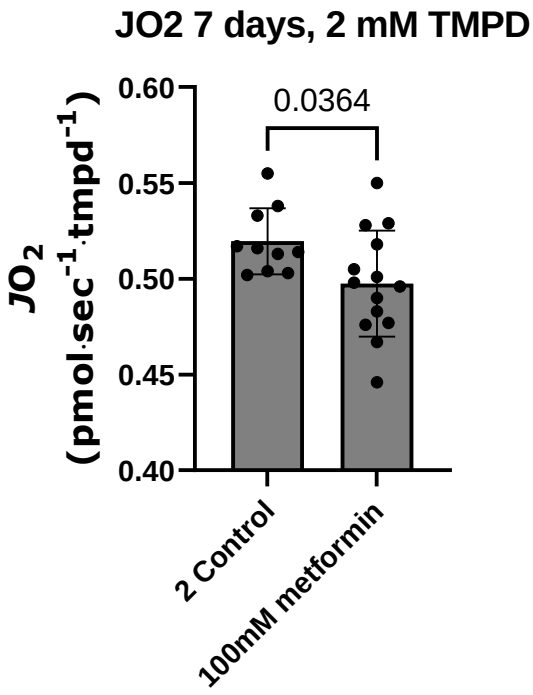
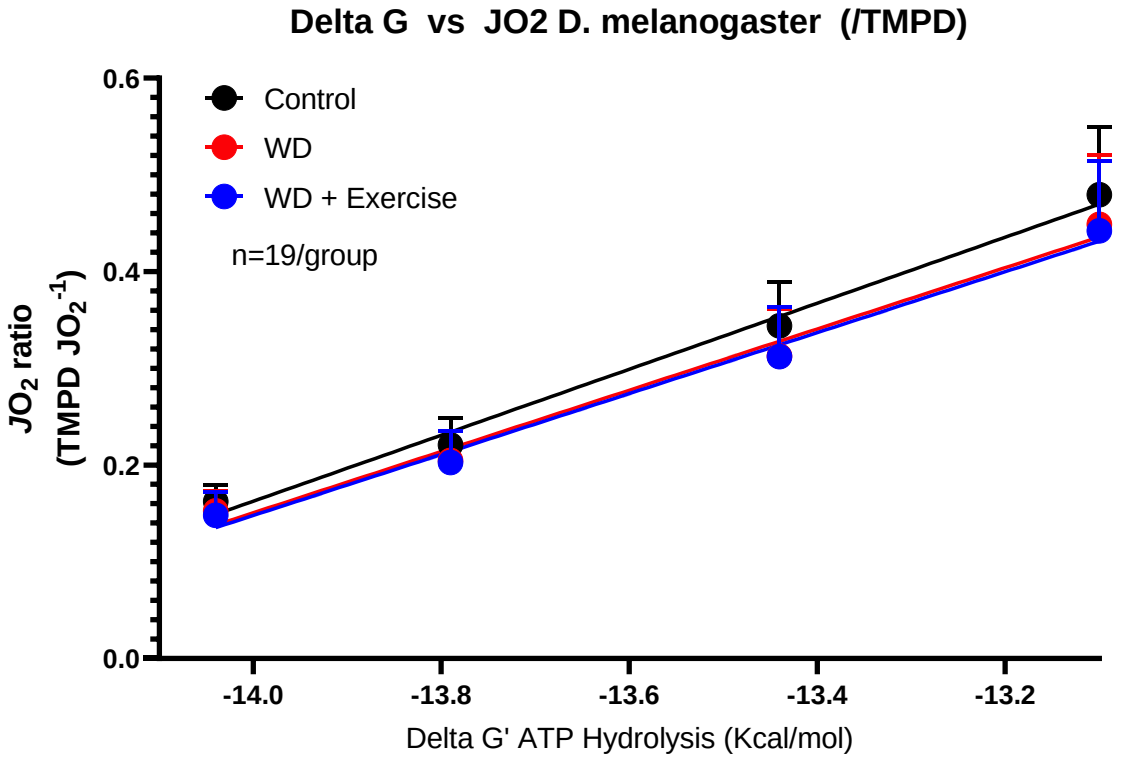


Figure 6. *In vivo* results from 7 days on metformin at 2 mM ADP rate normalized to TMPD data.

Western Diet Studies

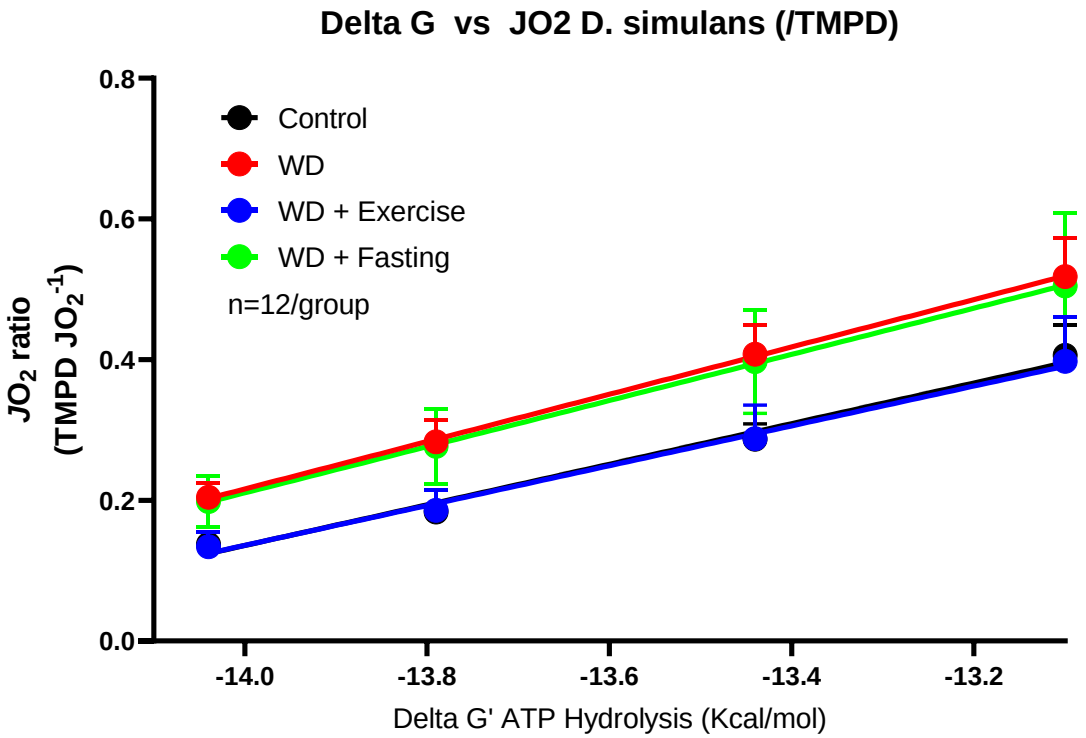
Mitochondrial respiration experiments showed that there was no difference in mitochondrial efficiency between the Control, Western Diet (high fat, sugar, and sodium), and Western Diet with Exercise in *D. melanogaster* (Fig. 7). Data for *D. melanogaster* was normalized to Complex IV activating TMPD data. These experiments were replicated with *D. simulans* to compare the two species as, although they are in the same melanogaster species subgroup, *D. simulans* have shorter life spans and since they are wild-caught flies, may have had more exposure to a low-fat diet. Data from *D. simulans* was normalized to both Complex IV activating TMPD data and wet tissue weight of the fly thorax cuticle. TMPD normalized data for *D. simulans* showed lower mitochondrial efficiency in WDE flies compared to the WD and WDF flies (Fig. 8). Data

normalized to wet tissue weight (mg) further showed a reduction of mitochondrial efficiency in WDE flies compared to WD flies (Fig. 9).



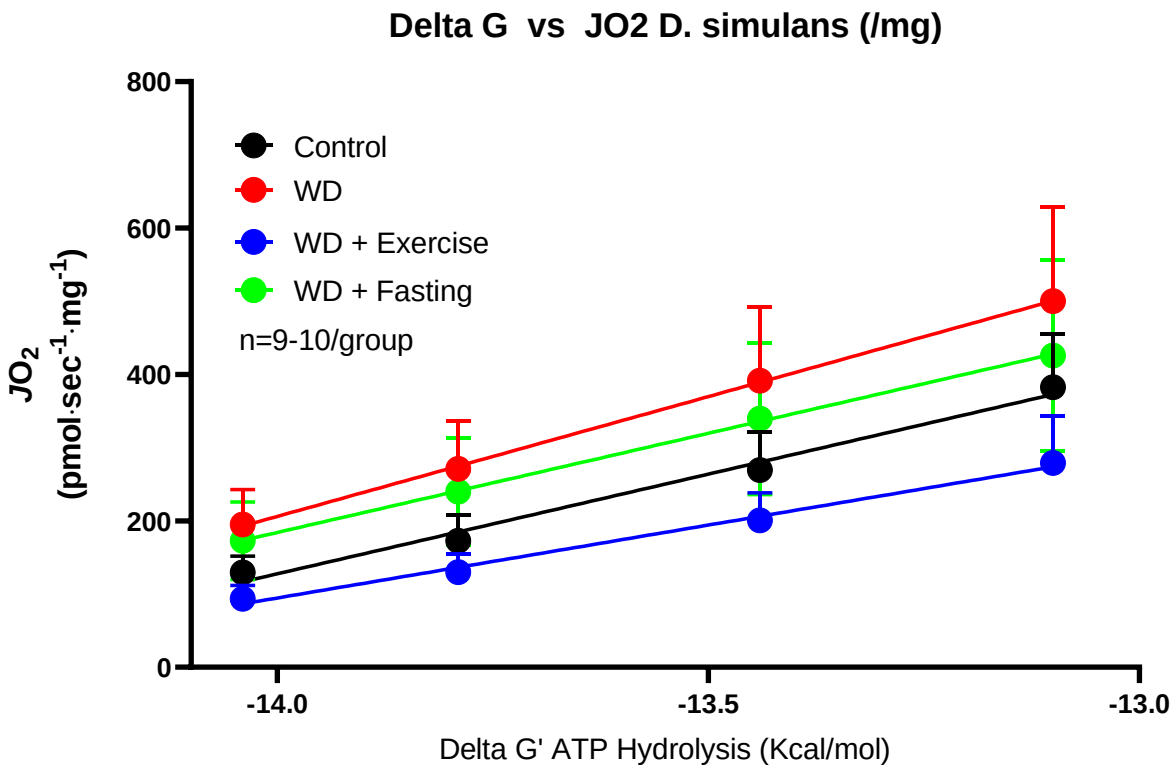
ForceFlow Multi in O2K: (mM)
2 Mal/ 5 Pyr/ 20U/ml CK/1 PCr + 10 ATP + PCr titr (3.75 + 7 + 13 + 20) + 0.005 Rot/ 0.005 AmA/ 2 Ascobate/ 0.5 TMPD.

Figure 7. Data for *D. melanogaster* normalized to Complex IV activating TMPD data**.**



ForceFlow Multi in O2K: (mM)
2 Mal/ 5 Pyr/ 20U/ml CK/1 PCr + 10 ATP + PCr titr (3.75 + 7 + 13 + 20) + 0.005 Rot/ 0.005 AmA/ 2 Ascobate/ 0.5 TMPD.

Figure 8. Data for *D. simulans* normalized to Complex IV activating TMPD data.



ForceFlow Multi in O2K: (mM)
2 Mal/ 5 Pyr// 20U/ml CK/1 PCr + 10 ATP + PCr titr (3.75 + 7 + 13 + 20) +
5 Malonate/0.005 AmA + 2 Ascobate + 0.5 TMPD.

Figure 9. Data for *D. simulans* normalized to wet tissue weight.

Discussion

In vivo Studies

In the *in vivo* studies, it was hypothesized that JO2 would decrease with higher mTPP and metformin concentrations as the cations should reduce the efficiency of the mitochondria. This was displayed in the *in vitro* experiments, but the same trends did not translate to *in vivo* experiments. This may be due to several factors including ineffective mTPP and metformin

ingestion/absorption, variability in data normalization results, and uncertainties with food preparation.

Experiments with mTPP only showed significant differences between basal and 0.050 mM ADP groups normalized to Complex IV activating TMPD data. The basal rate shows how much energy is used while there is no extra work strain on the mitochondria (exercise). The decreasing oxygen consumption rate for different mTPP concentrations at basal rate demonstrates how flies at on high concentrations of mTPP have less efficient mitochondria. Adding ADP stimulates the mitochondria to produce more ATP. However, the decrease in oxygen consumption was not consistent across the different concentrations of ADP added. These results are unclear as more ADP was added, higher concentrations of mTPP should have had lower JO2. In addition, more groups were found to have a significant decrease in JO2 after seven days compared to four hours and two hours (which found no significant difference). This suggests that two hours may not have been enough time for the mTPP to be absorbed by the gastrointestinal tract and circulated to the flight muscles. Although there was evidence that all the flies ingested the food (by the presence of blue dye), ADP-stimulated JO2 was surprisingly not affected evenly in all groups, suggesting that mTPP was not absorbed or that it is ineffective *in vivo*. Even if mTPP was absorbed into the gastrointestinal tract, it may be rendered inactive or unable to penetrate the cell, specifically the mitochondria. There is also evidence of ineffective mTPP when examining the survival rates of the flies on mTPP compared to metformin. With mTPP, there was a 100% survival rate across all groups, including the 1 mM mTPP “megadose” group. In contrast, metformin was much more lethal with a survival rate of 40% in the 500 mM and 1 M metformin groups after treating for 24 hours.

Data normalized to cuticle tissue wight data had more variability as tissue fluid evaporated within minutes. There was also variability with weighing the tissue on the microscale since the flies themselves only weighed around 0.100 to 0.300 mg. Moreover, during several experiments, the microscale had to be recalibrated and multiple flies had to be omitted from the normalized to weight data because of erroneous fly weight (<0.100 mg or >0.300 mg). This may have been due to the possible evaporation of tissue fluid while weighing. To limit the loss of tissue fluid, flies were weighed in the same buffer used in Oxygraph-2Ks which also prevented cation dissociation. In addition, to prevent loss of buffer, tubes were centrifuged before each weighing (first without the fly tissue and then with the fly tissue).

The food provided to the flies could also have reacted with mTPP and metformin, preventing the cations from entering the mitochondria. In addition, at high concentrations, metformin proved to be difficult to dissolve and had to be mildly heated to prevent crystalizing out of the diH2O. This constant heating may have altered its effectiveness. On the other hand, mTPP was more soluble and did not have to be heated to dissolve. To prepare the food, mTPP was first dissolved in diH2O and then added to freshly prepared standard Bloomington Formulation diet at ~70ºC before solidifying. While 70ºC is much lower than the melting point of mTPP, any heating may have altered the cation. Thus, later experiments changed to a diet that could be prepared at room temperature. However, changing the preparation of the food showed no difference in mTPP or metformin effect. Moreover, mTPP still did not cause any deaths even in the supposedly fatal dose of 1 M mTPP.

Western Diet Studies

When comparing *D. melanogaster* and *D. simulans,* mitochondrial efficiency of *D. melanogaster* was not impacted by the different experimental combinations while mitochondrial efficiency of *D. simulans* under WDE was decreased compared to the other experimental combinations. *D. melanogaster* may have not shown effects of the Western Diet because they have a longer life span which may have slowed the impacts of the diet. Since the *D. melanogaster* are also commercially purchased, they may be less susceptible to diet changes from transgenerational exposure to commercially available fly food. On the other hand, *D. simulans* were wild caught and may not have transgenerational exposure to food high in fat, sugar, and salt content. Interestingly, and in opposition to preliminary data, *D. simulans* under WDE had a decreased mitochondrial efficiency. This may be because the flies in this study did not exercise as much as the previous study; therefore, the treatment of exercise was insufficient. The mitochondrial efficiencies may depend on the climate or season during which the original stock flies were caught. In addition, the population of *D. simulans* consists of genetically heterogeneous individuals whose genetic, physiological, and bioenergetic repertoire has been altered by being predisposed to exterior influences such as different "real world" diets and climate. Further investigations will be required to understand these results.

Another limitation to the amount of data collected, especially for *D. simulans,* was the low survival rate once the flies were put on the Western Diet. In fact, the data for *D. simulans* on WD and WDF had to be repeated because only one out of twenty WD flies survived the five days on the diet and only five out of the twenty WDE flies survived. In addition, the flies that did survive appeared sluggish and had low basal mitochondrial rates. Thus, the data collection for WD and

WDE was repeated to have twelve data points per group. This further shows the toxicity of the WD.

Due to its greater effect on JO2 and survival rate compared to mTPP, metformin will be used in future investigations to determine its impact on mitochondrial efficiency and lifespan. Future investigations will also address how diet and mitochondrial efficiency combinations relate to lifespan and if they affect the mitochondrial efficiency of offspring.

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