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The Effects of a “Mito-Cocktail” on Acute Galactose Sensitivity and Long-Term Outcomes of *GALT−/−* *Drosophila melanogaster*

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## Abstract

The Effects of a "Mito-Cocktail" on Acute Galactose Sensitivity and Long-Term Outcomes of *GALT–/–*

*Drosophila melanogaster*

By Hannah Schwartz

Classic Galactosemia (CG) is an autosomal recessive metabolic disorder that results from impaired metabolism of galactose due to profound deficiency of the enzyme galactose-1-phosphate uridylyltransferase (GALT). After exposure to milk (which contains high levels of galactose), acute symptoms appear and intensify rapidly. Early identification by Newborn Screening and dietary restriction of galactose minimizes acute outcomes of the disease. However, long-term outcomes remain an enormous burden for CG patients. At this time, there is no known intervention to prevent or reverse long-term effects of CG, largely due to the fact that the underlying pathophysiology of long-term outcomes in CG remains unknown. It has been identified that GALT-null *Drosophila* exhibit galactose sensitivity as larvae. Previous studies have also shown that oxidative stress likely plays a role in galactosemia and that two antioxidants, Vitamin C and α-mangostin, increase survival of galactose-exposed *GALT–/– Drosophila* larvae. Prior studies also suggest mitochondrial fuction may be impaired in GALT definciency. As an attempt to alleviate the symptoms associated with CG, we have directly tested a set of nine food additives predicted to counter mitochondrial deficiency. We have done this by using a *Drosophila melanogaster* model and have identified a number of pharmacological modifiers, a "mito-cocktail," that may affect outcomes of *GALT–/– Drosophila*. The mito-cocktail includes: alpha lipoic acid (ALA), carnitine, cobalamin, folic acid, nicotinamide, pyridoxine, riboflavin, thiamine, and coenzyme Q10. Previous data from our lab have showed that addition of the full "mito-cocktail" to dex + gal fly food partially rescued the survival of *GALT –/–* larvae to pupation and produced an increase in the survival rates of mutant larvae to adulthood. We confirmed this finding and also found that a specific subset of the "mito-cocktail" components consisting of ALA, cobalamin, folic acid, pyridoxine, and thiamine improved acute outcomes on its own. Further, we found that cobalamin alone improved acute outcomes. The effect of the "mito-cocktail" on long-term outcomes remains unknown.

# The Effects of a "Mito-Cocktail" on Acute Galactose Sensitivity and Long-Term Outcomes of *GALT−/−* *Drosophila melanogaster*

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## Acknowledgements

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## INTRODUCTION

Classic Galactosemia (CG) is an autosomal recessive metabolic disorder that results from impaired metabolism of galactose due to deficiency of the enzyme galactose-1-phosphate uridylyltransferase (GALT)[1].

**Figure 1** depicts the Leloir pathway of galactose metabolism. CG occurs in about 1/50,000 births in the United States[2] and affected newborns appear mostly normal[1]. After exposure to milk (which contains high levels of galactose), acute symptoms can appear and intensify rapidly. Although the majority of the severe symptoms can be prevented or treated, many patients still suffer from long-term complications including cognitive, speech, and behavioral problems, motor disabilities, growth deficiencies, and among girls and women, primary or premature ovarian insufficiency (POI).

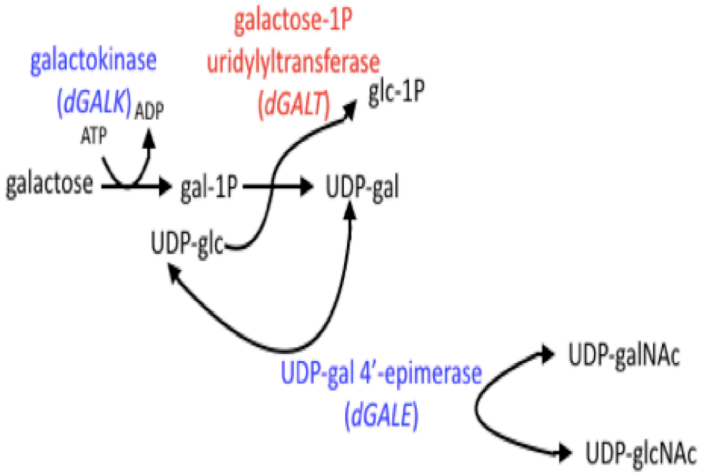


**Figure 1**: The Leloir pathway of galactose metabolism. Classic Galactosemia results from impairment of galactose-1P uridylyltransferase (*dGALT)*, shown in red. *dGALT* catalyzes the synthesis of glucose-1P and UDP-galactose from galactose-1P and UDP-glucose. Other enzymes in the pathway are shown in blue.

CG is one of the most common metabolic disorders identified by newborn screening (NBS) in the US[2]; rapid identification by NBS and dietary restriction of galactose minimizes acute outcomes of the disease. However, long-term outcomes remain an enormous burden for CG patients. At this time, there is no known intervention to prevent or reverse these long-term effects of CG. This is largely due to the reality that the underlying pathophysiology of long-term

outcomes in CG remains unknown. A number of hypotheses have been suggested, but none confirmed.

Prior work has demonstrated that GALT-null *Drosophila* (AP2) exhibit galactose sensitivity as larvae. **Figure 2**[3] shows 1) that galactose exposure is lethal to *GALT−/− Drosophila* larvae and that galactose restriction rescues survival and 2) that *Drosophila* fail to survive to adulthood if exposed to moderate levels of galactose (glc+gal), but survive if maintained on food lacking galactose (glc). **Figures 3a** and **3b** depict pupal survival and adult survival, respectively, of *GALT+/+ Drosophila* (C2) and *GALT−/− Drosophila* raised on both dextrose food (dex) and 200mM galactose food (gal); *GALT−/− Drosophila* demonstrate significantly decreased survival on 200mM galactose food to both pupal and adult stages.

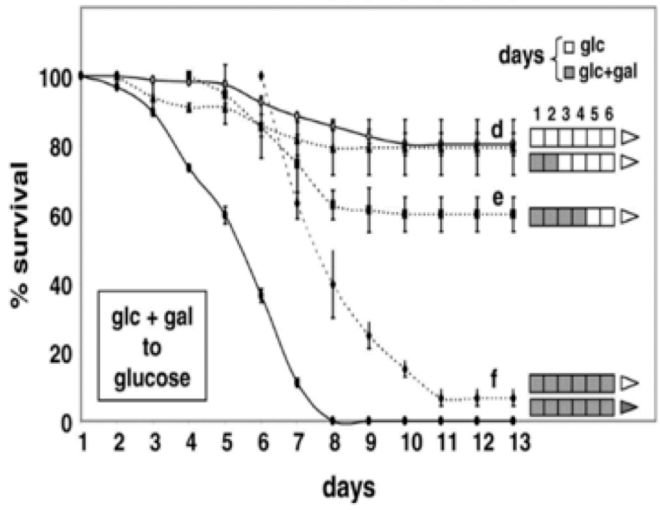


**Figure 2:** Galactose (gal) exposure is lethal to *GALT-/- Drosophila* larvae galactose restriction rescues survival. (Kushner et al. 2010). Reproduced with permission from the journal.

**Figure 4**[3] shows a galactose-independent movement defect in adult GALT-null *Drosophila* as measured by climbing[4] in a counter-current device. **Figure 5** further depicts a galactose-independent movement defect in adult GALT-null *Drosophila* as measured using a simple climbing assay. Previous studies have also shown that oxidative stress likely plays a role in galactosemia and that two antioxidants, Vitamin C, (**Figure 6**[5]), and α-mangostin (**Figure 7**[5]),

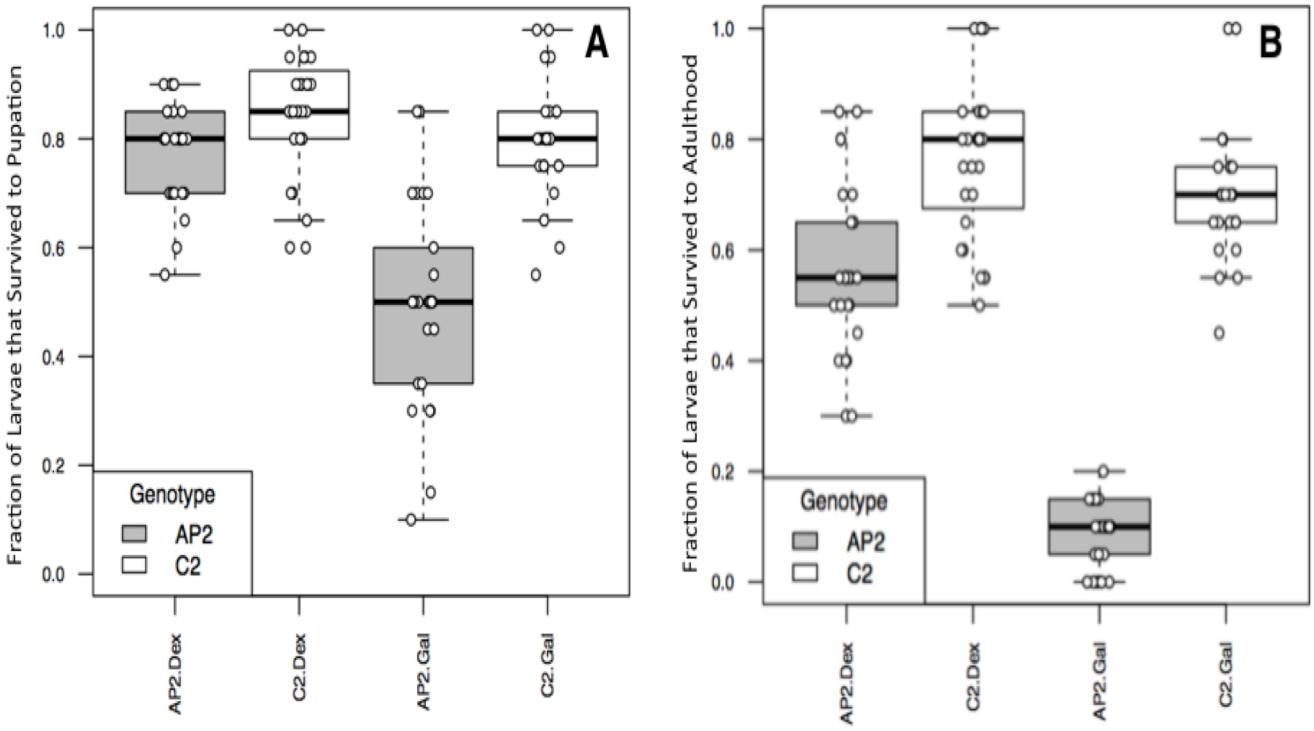


**Figure 3:** Survival to pupation (**A**) and adulthood (**B**) of *GALT +/+* (white, C2) and *GALT -/-* (grey, AP2) *Drosophila* raised on either dextrose food or dextrose food supplemented with 200mM galactose. A significant negative effect of galactose supplementation on the survival rates of GALT-null larvae to pupation (**A**) ($p<0.00001$, Fisher's Exact Test) and adulthood (**B**) ($p<0.00001$, Fisher's Exact Test) can be seen. Dex= food containing 555mM dextrose, Gal= food containing 555mM dextrose + 200mM galactose

both increased survival of galactose-exposed *GALT–/– Drosophila melanogaster* larvae,[5] at least at some concentrations.

Seeking dietary modifiers to alleviate the acute and long-term consequences associated with GALT-deficiency, we have directly tested a set of nine food additives predicted to counter the metabolic consequences of mitochondrial deficiency that we hypothesize may contribute to pathophysiology in CG. We have done this using a GALT-null *Drosophila melanogaster* model of CG. The results of these experiments may give an evidence-based approach to intervention for complications in CG. In fact, many of the complications that CG patients experience are also present in patients with other diseases. If we can identify a potential intervention to the metabolic complications suffered by CG patients, we may also be able to generalize these findings to benefit patients with other conditions. Toward this end, we have identified a

number of pharmacological modifiers that may affect the outcomes of *GALT–/– Drosophila* (**Table 1**). These compounds are explained below.

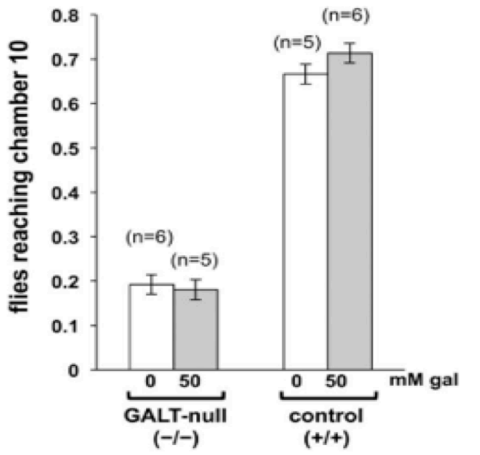


**Figure 4:** *GALT -/- Drosophila* demonstrate a climbing defect independent of galactose exposure. (Ryan et al. 2012) Reproduced with permission from the journal.

Alpha lipoic acid (ALA), a known antioxidant, is both water and fat soluble and synthesized in the human body[6]. As an antioxidant, this compound helps to rid the body of free radicals. ALA is used in treating deficits in the central and peripheral nervous systems in diabetes patients[7,8] and functions in mitochondrial dehydrogenase reactions, ridding the body of toxic aldehydes.

L- carnitine, a water-soluble ammonium compound, is present at high levels in the liver, skeletal muscle, heart and kidney[9]. L-carnitine plays a large role in transporting long-chain fatty acids across the mitochondrial membrane to facilitate beta-oxidation, part of the process that converts fat into sugar[10]. L-carnitine is also thought to play a role in ridding the mitochondria of toxic compounds, such as 3-nitropropionic acid (a mitochondrial inhibitor), preventing their accumulation[11]. Its antioxidant and anti-inflammatory characteristics have been hypothesized to protect against Ischemia Reperfusion Injury (IRI)[12], tissue damage due to transient lack of

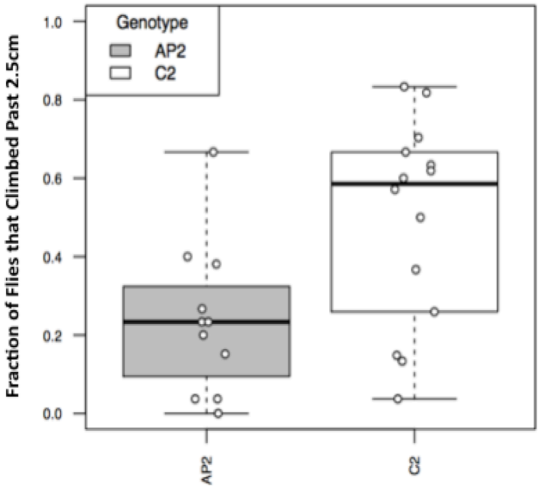


**Figure 5:** *GALT -/- Drosophila* (AP2) demonstrate a significant climbing defect despite dietary galactose restriction. ($p<0.00001$, Fisher's Exact Test)

oxygen. Water-soluble B vitamins[13], specifically, cobalamin ($B_{12}$), folic acid ($B_9$), nicotinamide ($B_3$), pyridoxine ($B_6$), riboflavin ($B_2$), thiamine ($B_1$), may play roles modifying long-term outcomes outcomes of *GALT−/−* *Drosophila*. Cobalamin, nicotinamide, and thiamine contribute to glucose production and carbohydrate metabolism. Cobalamin, folic acid, pyridoxine, and thiamine assist in nervous system function[14,15,16]. Specifically, cobalamin and folic acid may have roles in preventing disorders of CNS development, mood disorders, and dementias, such as Alzheimer's disease[14]. Pyridoxine acts as

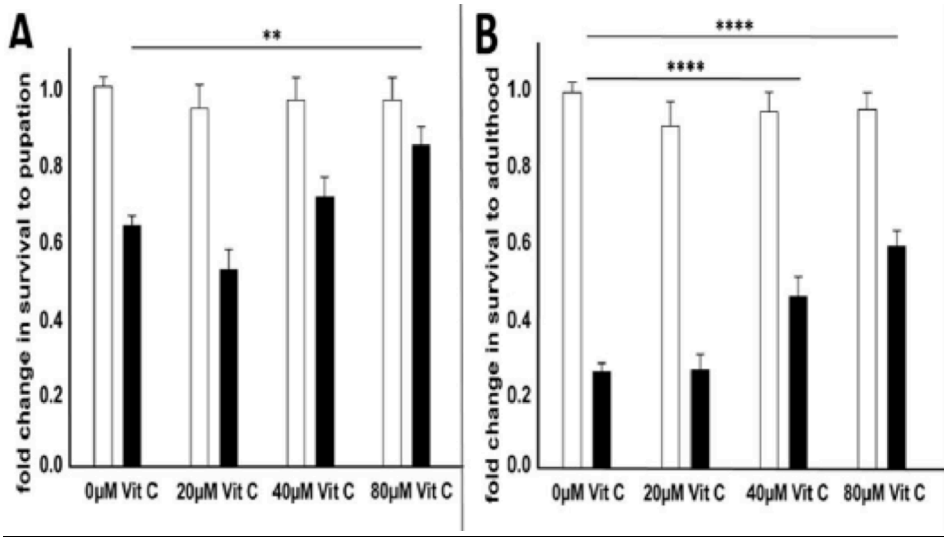


**Figure 6**: Survival to pupation (**A**) and adulthood (**B**) of *GALT +/+* (white) and *GALT -/-* (black) *Drosophila* supplemented with Vitamin C. A significant positive impact of Vitamin C treatment on the survival rates of GALT-null larvae exposed to galactose can be seen. (Jumbo-Lucioni et al. 2013) Reproduced with permission from the journal.

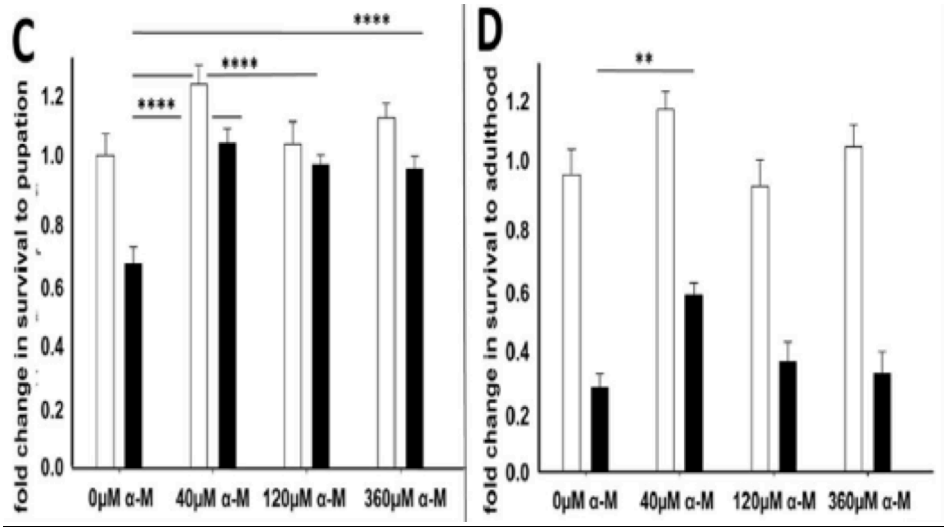


**Figure 7**: Survival to pupation (**C**) and adulthood (**D**) of *GALT +/+* (white) and *GALT -/-* (black) *Drosophila* supplemented with α-mangostin. A significant positive impact of α-mangostin treatment on the survival rates of GALT-null larvae exposed to galactose can be seen. (Jumbo-Lucioni et al. 2013) Reproduced with permission from the journal.

| Pharmacological Agent | Facts and Functions | Pathways Involved |
|---|---|---|
| Alpha Lipoic Acid[6, 7,8] | • Plays an essential role in mitochondrial dehydrogenase reactions<br>• Rids free radicals<br>• Protects membranes by interacting with vitamin C and glutathione, which may in turn recycle vitamin E<br>• Shown to be beneficial in a number of oxidative stress models<br>• Fat and water soluble, can pass through blood-brain barrier | • Antioxidant<br>• Nervous system function |
| L-Carnitine[9, 10, 11, 12] | • Transports long-chain fatty acids across the mitochondrial membrane to facilitate beta-oxidation, turning fat into sugar<br>• Protective role against various mitochondrial toxins<br>• More abundant in athletes<br>• Antioxidant and anti-inflammatory effects<br>• Water soluble<br>• Present in liver, skeletal muscle, heart, and kidney | • ATP Production<br>• Antioxidant |
| Cobalamin[13, 14, 17] (Vitamin $B_{12}$) | • Assists in glucose production<br>• Maintains healthy nerve cells<br>• Deficiency can lead to immune system malfunction<br>• Prevents disorders of CNS development, mood disorders, and dementias<br>• Essential for nucleotide synthesis and genomic and non-genomic methylation.<br>• Water soluble | • Nervous system function<br>• Immune system function<br>• Vitamin digestion/ absorption |
| Coenzyme Q10[19] | • Powerful antioxidant<br>• Essential in oxidative phosphorylation and therefore ATP synthesis<br>• Fat soluble | • ATP production<br>• Antioxidant |
| Folic Acid[13, 14, 17, 18] (Vitamin $B_9$) | • Deficiency leads to central nervous system complications<br>• Prevents disorders of CNS development, mood disorders, and dementias<br>• Essential for nucleotide synthesis and genomic and non-genomic methylation.<br>• Deficiency affects T cell-mediated immunity<br>• Not found in the body<br>• Water soluble | • Nervous system function<br>• Immune system function<br>• Vitamin digestion/ absorption |
| Nicotinamide[13] (Vitamin $B_3$) | • Plays a role in carbohydrate metabolism<br>• End product of tryptophan metabolism<br>• Precursor to NAD<br>• Essential coenzyme in ATP synthesis<br>• Water soluble | • ATP production<br>• Vitamin digestion/ absorption |
| Pyridoxine [13, 16] (Vitamin $B_6$) | • Necessary for proper CNS development<br>• Acts a coenzyme in the biosynthesis of the neurotransmitters GABA, dopamine and serotonin<br>• Protective effects to atherosclerosis and ischemia-reperfusion injury (IRI)<br>• Cannot be synthesized in the human body<br>• Water soluble | • Nervous system function<br>• Immune system function<br>• Vitamin digestion/ absorption |
| Riboflavin[13, 17] (Vitamin $B_2$) | • Deficiency affects T cell-mediated immunity<br>• Works as antioxidant by destroying free radicals<br>• Water soluble | • Antioxidant<br>• Immune system function<br>• Vitamin digestion/ absorption |
| Thiamine[13, 15] (Vitamin $B_1$) | • Deficiency affects the central nervous system, peripheral nervous system, and cardiovascular system<br>• Plays a role in carbohydrate metabolism<br>• Cofactor in macronutrient metabolism<br>• Plays a role in nerve structure and function as well as brain metabolism<br>• Used as a therapeutic agent in patients with diabetes<br>• Water soluble | • Nervous system function<br>• ATP production<br>• Vitamin digestion absorption |

**Table 1:** Basic functions and pathways involved for 9 different pharmacological agents to be used in this study.

a coenzyme in the biosynthesis of many important neurotransmitters including GABA, dopamine, and serotonin[16]. Riboflavin, folic acid, and cobalmin are also shown to affect immune system function,[17, 18] specifically T cell-mediated immunity. Nicotinamide and thiamine both act in ATP synthesis, and riboflavin has strong antioxidant properties.

Lastly, coenzyme Q10 (CoQ10), a fat-soluble coenzyme also known as ubiquinone, is a key player in oxidative phosphorylation in mitochondria—a process that makes ATP—and is also shown to be a strong antioxidant[19]. Together, all nine of these compounds constitute what we call the "mito-cocktail."

The components of the "mito-cocktail" can be further classified into 5 smaller groups consisting of 4-5 compounds each (**Table 2**). By examining the acute and long-term effects of each component and functional grouping of the "mito-cocktail" components on GALT-null *Drosophila*, we can explore whether one or more of these compounds, or potential groupings, might help to minimize the complications resulting from GALT deficiency

| **Group** | **Contents** |
|---|---|
| Antioxidants | Alpha Lipoic Acid, Carnitine, Coenzyme Q10, Riboflavin (Vitamin $B_2$) |
| Nervous System Function | Alpha Lipoic Acid, Cobalamin (Vitamin $B_{12}$), Folic Acid (Vitamin $B_9$), Pyridoxine (Vitamin $B_6$), Thiamine (Vitamin $B_1$), |
| Immune System Function | Cobalamin (Vitamin $B_{12}$), Folic Acid (Vitamin $B_9$), Riboflavin (Vitamin $B_9$), Pyridoxine (Vitamin $B_6$) |
| Vitamin digestion/absorption | Cobalamin (Vitamin $B_{12}$), Folic Acid (Vitamin $B_9$) Nicotinamide (Vitamin $B_3$), Riboflavin (Vitamin $B_2$), Thiamine (Vitamin $B_1$) |
| ATP production | Carnitine, Coenzyme Q10, Nicotinamide (Vitamin $B_3$), Thiamine (Vitamin $B_1$) |

**Table 2.** "Mito-cocktail" components divided into 5 functional groups.

## MATERIALS AND METHODS:

### Fly Stocks and Maintenance:

Fly stocks were maintained at 25° Celsius with 60-70% humidity on a molasses-based food containing 43.5 g/L cornmeal, 17.5 g/L yeast extract, 8.75 g/L agar, 54.7 mL/L molasses, 10 mL propionic acid and 14.4 mL/L tegosept mold inhibitor (10% w/v in ethanol). The AP2 and C2 fly stocks were generated in the Fridovich-Keil lab[3]. All other fly stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University unless otherwise noted.

### Food Preparation:

Food for survival assays: Dextrose and dextrose + galactose foods were prepared with 5.5 g/l agar, 40 g/l yeast, 90 g/l cornmeal, 100 g/l dextrose, 10 ml/l propionic acid and 14.4 ml/l tegosept mold inhibitor (10% w/v in ethanol). For testing the effect of the full "mito-cocktail," two stocks were prepared, one of water-soluble components (carnitine, thiamine, riboflavin, nicotinamide, pyroxidine, and folic acid), and one of ethanol-soluble components (cobalamin, ALA, and CoQ10). Once the food was at room temperature, each "mito-cocktail" stock was added to fly food by a 1:20 dilution such that the final concentrations of the components in the food were: 42mM carnitine, 0.06mM thiamine, 0.05mM riboflavin, 4.9mM nicotinamide, 0.1 mM pyroxidine, 0.1mM folic acid, 0.1mM cobalamin, 1.9mM ALA, and 3.8mM CoQ10. These concentrations were determined from literature and previous experimentation in our lab. For testing the effect of the functional groupings and individual components, stocks were prepared in the same manner containing their respective components, and added to food in the same

final concentrations. Control food was prepared by adding water and ethanol in the same volumes as "mito-cocktail" to the food. All food was dispensed into 0.5mL Eppendorf tubes to solidify.

Food for climbing assays: Molasses-based food containing 43.5 g/L cornmeal, 17.5 g/L yeast extract, 8.75 g/L agar, 54.7 mL/L molasses, 10 mL propionic acid and 14.4 mL/L tegosept mold inhibitor (10% w/v in ethanol) was melted by heating in a microwave oven. Two "mito-cocktail" stocks were prepared in the same manner as above and added to the melted and cooled (to room temperature) molasses-based food at a 1:20 dilution such that the final concentrations of the components in the food were: 42mM carnitine, 0.06mM thiamine, 0.05mM riboflavin, 4.9mM nicotinamide, 0.1 mM pyroxidine, 0.1mM folic acid, 0.1mM cobalamin, 1.9mM ALA, and 3.8mM CoQ10. For testing the effect of the functional groupings, stocks were prepared in the same manner containing their respective components, and added to food to the same final concentrations. Control food was prepared by adding water and ethanol in the same amounts as "mito-cocktail" to the food. All food was dispensed into standard 25mm diameter by 95mm high *Drosophila* vials to solidify.

**Survival assays:**

Survival assays assessed how many *Drosophila* larvae live from L1 to the pupal and adult stages. 0.5mL Eppendorf tubes containing fly food prepared as described above were loaded with twenty L1 larvae per tube. The caps of the Eppendorf tubes were cut off and each tube was placed in a 5mL Polystyrene Round-Bottom Tube. Each 5mL tube was then capped with a cotton ball and placed at 25° Celsius. Approximately eight to ten days later the number of

pupae in each tube were counted, and up to eighteen days later eclosing adults were also counted.

**Climbing assays:**

Climbing assays have been previously used to quantify a long-term movement defect in our GALT-null flies[4,20]. Standard *Drosophila* vials described above were loaded with between fifty and one hundred L1 larvae and placed at 28° Celsius. Newly-eclosed flies were retrieved from the vials and sorted by gender and then tapped into new vials and incubated for two days at 28° Celsius. After the two-day maturation period, the flies were transferred to 18x150mm glass tubes for testing; each tube had a line marked 2.5cm from the bottom of the tube. These tubes were capped with cotton and dropped individually from a fixed height, down a chute onto a pad on the bench, knocking all the flies to the very bottom of the tube. The number of flies that climbed back up the sides of the tube past the 2.5cm line within ten seconds of the tube hitting the bench was counted and recorded. Replicate tubes of each genotype and environmental condition were tested, and controls were always tested in parallel with GALT-null flies to avoid potential artifacts from subtle differences in environment or testing that might occur (e.g. ambient temperature or humidity in the room).

**Statistical Analysis:**

Data were analyzed using Microsoft Excel and R. The mean, standard deviation, and standard error of the mean (SEM) were calculated for each genotype and exposure group in each trial. P-values were also calculated, using Fisher's Exact Test and Chi-Square Tests of

Independence, with $p<.05$ being the cut-off for significance. Post-hoc analysis was also performed when neccesary, making the proper adjustments for multiple test corrections (Bonferroni Correction).

## RESULTS

### Acute Outcomes:

We tested the effects of the full "mito-cocktail" on the survival of GALT-null and control *Drosophila* larvae deposited on fly food containing either 555mM dextrose (dex) or dextrose plus 200mM galactose (dex + gal). We analyzed the data using Fisher's Exact Test and found that addition of the full "mito-cocktail" to dex + gal food partially rescued the survival of GALT-null larvae to pupation ($p<0.00001$) and produced an increase in the survival rates of mutant larvae to adulthood ($p<0.00001$) (**Figure 8)**. Additionally, we found that addition of the "mito-cocktail" to dex + gal food increased survival of GALT-null larvae to adulthood by 26.45%.

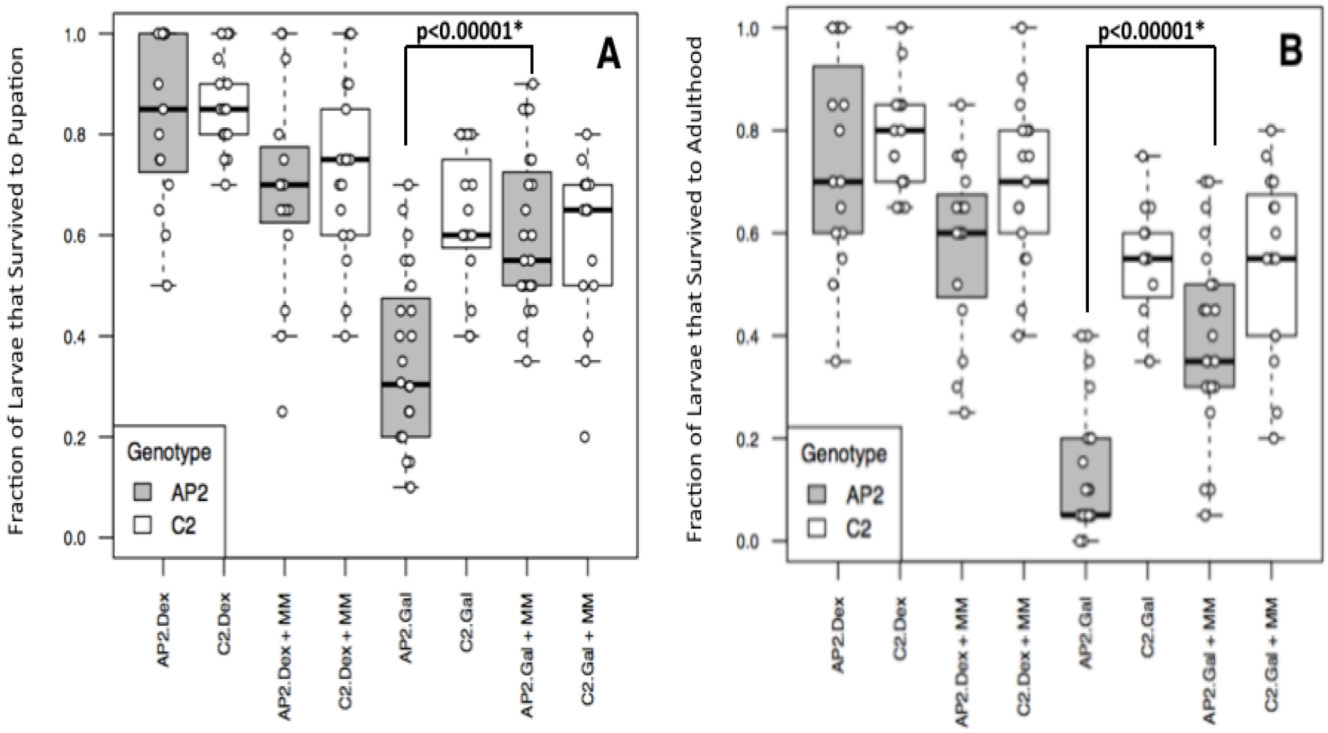


**Figure 8**: Survival to pupation (**A**) and adulthood (**B**) of *GALT +/+* (white, C2) and *GALT -/-* (grey, AP2) *Drosophila* supplemented with the "mito-cocktail." A significant positive impact of the "mito-cocktail" treatment on the survival rates to pupation (**A**) ($p<0.00001$, Fisher's Exact Test) and to adulthood (**B**) ($p<0.00001$, Fisher's Exact Test) of GALT-null larvae exposed to galactose can be seen. Dex= food containing 555mM dextrose, Gal= food containing 555mM dextrose + 200mM galactose, MM= food containing 555mM dextrose + 200mM galactose + "mito-cocktail."

We then set out to determine if specific functional groupings of the full "mito- cocktail," shown in **Table 2**, had an effect on acute galactose sensitivity (**Figure 9**). We used a Chi-Square Analysis with five degrees of freedom to analyze the data in **Figure 9**, and found a significant effect of the five functional groupings on acute survival of GALT-null *Drosophila* when raised on galactose to pupation (**Figure 9a**, p=0) and to adulthood (**Figure 9b**, p=0). **Table 3** presents all pairwise p-values obtained from post-hoc Chi-Square Analysis with a Bonferroni adjusted significance level of 0.0033.

While the addition of the Nervous System Function group to 200mM galactose food was the only group that showed a significant positive effect on acute galactose sensitivity to both pupation (p=0) and to adulthood (p=8.00E-08) when compared with dex + gal food alone, one

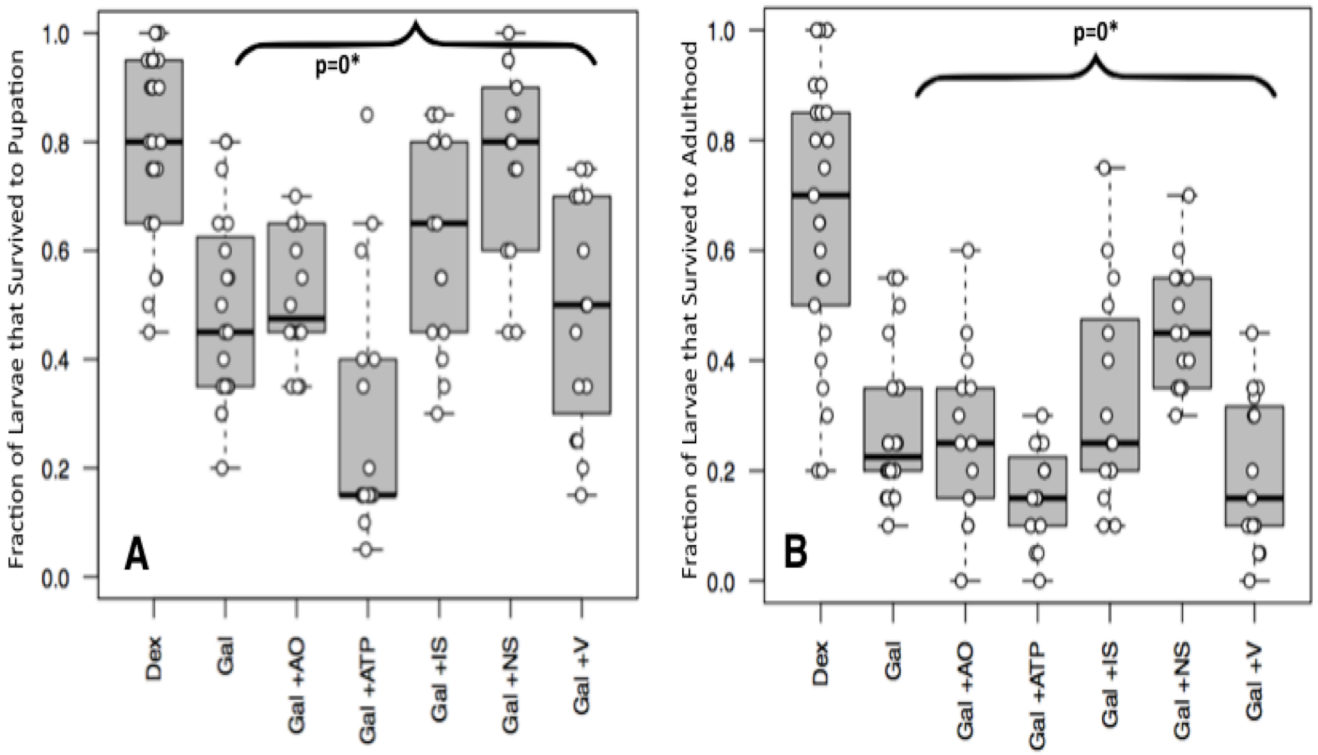


**Figure 9**: Survival to pupation (**A**) and adulthood (**B**) of *GALT -/-* (AP2) *Drosophila* supplemented with specific functional groupings (Table 2) of the "mito-cocktail." A significant positive impact of the functional groupings on the survival rates to pupation (**A**) (p=0, Chi Squared Analysis, α=.05) and to adulthood (**B**) (p=0, Chi Squared Analysis, α=.05) of GALT-null larvae exposed to galactose can be seen. Dex=food containing 555mM dextrose, Gal=food containing 555mM dextrose + 200mM galactose, AO=dex+ gal + Antioxidant functional group, ATP=dex+ gal + ATP Production functional group, IS=dex+ gal + Immune System Function functional group, NS=dex + gal + Nervous System Function functional group, V=dex+ gal + Vitamin Digestion and Absorption functional group.

| Comparison<br>Dex + Gal (n=480)<br>Dex + Gal + IS (n=300)<br>Dex + Gal + V (n=292)<br>Dex + Gal + ATP (n=300)<br>Dex + Gal + NS (n=280)<br>Dex + Gal + AO (n=280) | Increased vs. Decreased Survival to Adulthood Compared to Dex + Gal | % Increase or Decrease in Survival to Adulthood Compared to Dex + Gal | P-value Survival to Pupation | P-value Survival to Adulthood |
|---|---|---|---|---|
| Dex + Gal vs. Dex + Gal + IS | No Effect | 6.04 | 0.00024501** | 0.07216837 |
| Dex + Gal vs. Dex + Gal + V | No Effect | -13.43 | 0.73012161 | 0.00000111** |
| Dex + Gal vs. Dex + Gal + ATP | Decrease | -11.29** | 3.10E-07** | 0.00026371** |
| Dex + Gal vs. Dex + Gal + NS | Increase | 19.14** | 0** | 8.00E-08** |
| Dex + Gal vs. Dex + Gal + AO | No Effect | -1.22 | 0.50091373 | 0.71432023 |
| Dex + Gal + IS vs Dex + Gal + V | -- | -- | 0.00031551** | 0** |
| Dex + Gal + IS vs. Dex + Gal + ATP | -- | -- | 0** | 8.40E-07** |
| Dex + Gal + IS vs. Dex + Gal + NS | -- | -- | 0.00017455** | 0.00127457** |
| Dex + Gal + IS vs. Dex + Gal + AO | -- | -- | 0.00794425 | 0.05610289 |
| Dex + Gal + V vs. Dex + Gal + ATP | -- | -- | 0.00001599** | 0.4291953 |
| Dex + Gal + V vs. Dex + Gal + NS | -- | -- | 0** | 0** |
| Dex + Gal + V vs. Dex + Gal + AO | -- | -- | 0.36198353 | 0.0000597** |
| Dex + Gal + ATP vs. Dex + Gal + NS | -- | -- | 0** | 0** |
| Dex + Gal + ATP vs. Dex + Gal + AO | -- | -- | 2.30E-07** | 0.00285483** |

**Table 3.** Pairwise comparisons between all the groups shown in **Figure 9**. Column 1 states the groups being compared. Column 2 states the survival effect of the functional group on galactose exposed larvae. Column 3 reports the percent increase or decrease of larvae survival to adulthood in the presence vs. absence of the additives. Columns 4 and 5 report p-values of comparisons of survival to the pupal and adult stages, respectively. All p-values were calculated via post-hoc Chi-Square Analysis, α=.0033. **indicates statistical significance. Abbreviations explained in **Figure 9** legend.

group showed a significant negative effect. Specifically, addition of the ATP Production group to 200mM galactose showed a significant negative effect on survival of larvae to both pupation (p=3.10E-07) and adulthood (p=0.00026371) when compared with larvae deposited on dex + gal food alone. Further, addition of the Immune System Function group to dex + gal fly food significantly improved survival to pupation (p=0.00024501), but not to adulthood (p=0.07216837), and the Vitamin Digestion and Absorption group significantly decreased

survival to adulthood (p=0.00000111), but not to pupation (p=0.73012161). Lastly, the Antioxidant functional group showed no significant effect on survival of galactose exposed larvae to either pupation (p=0.50091373) or to adulthood (p=0.71432023) when compared to survival on dex + gal food alone. **Table 3** also shows the percent increase or decrease (positive or negative, respectively) of survival of galactose exposed GALT-null larvae to adulthood for each functional group.

As the Nervous System Function group appeared to have a significant effect on acute survival of galactose exposed larvae, we tested each of the components of the Nervous System Function group (ALA, Cobalamin, Folic Acid, Pyridoxine, and Thiamine) on survival of GALT-null

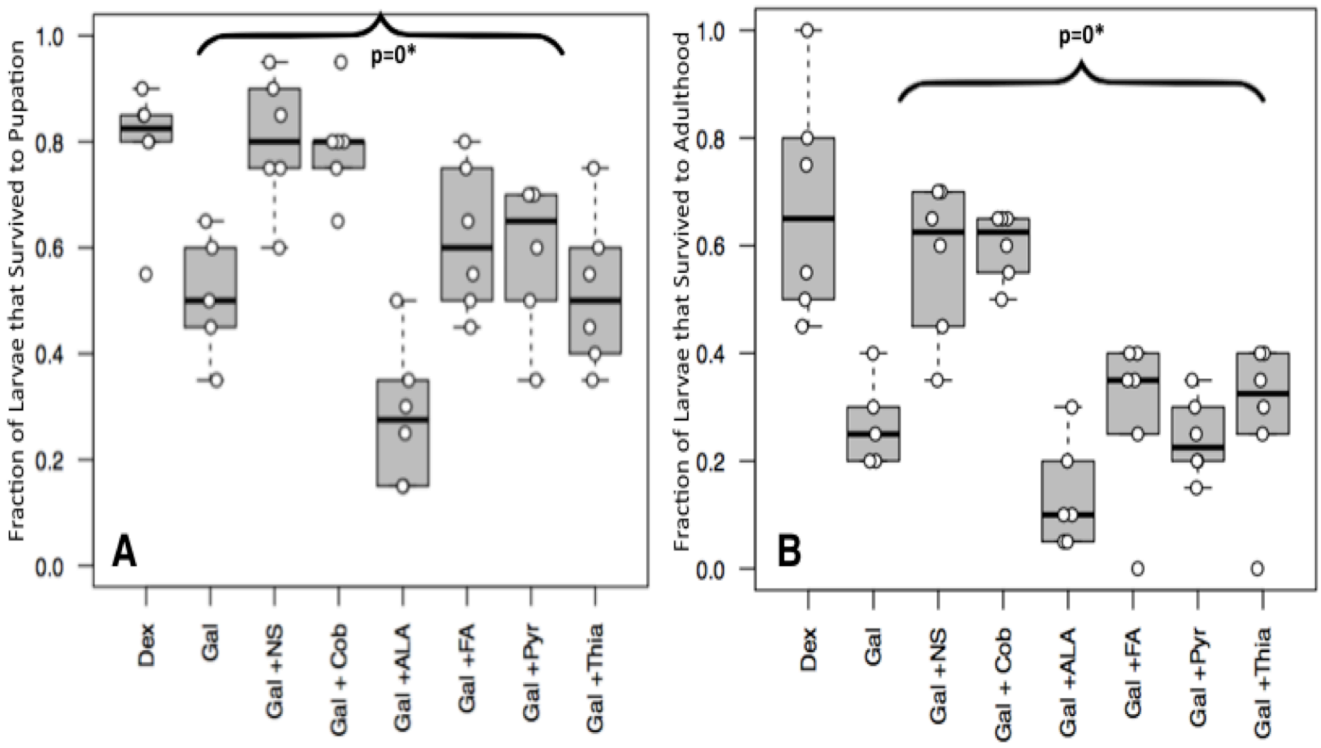


**Figure 10**: Survival to pupation (**A**) and adulthood (**B**) of *GALT -/-* (AP2) *Drosophila* supplemented with one compound each from the Nervous System Function group (Table 2). A significant positive impact of the individual compounds on the survival rates to pupation (**A**) (p=0, Chi Squared Analysis, α=.05) and to adulthood (**B**) (p=0, Chi Squared Analysis, α=.05) of GALT-null larvae exposed to galactose can be seen. Dex=food containing 555mM dextrose, Gal=food containing 555mM dextrose + 200mM galactose, NS=food containing 555mM dextrose + 200mM galactose + Nervous System function functional group, Cob= food containing 555mM dextrose + 200mM galactose + cobalamin, ALA= food containing 555mM dextrose + 200mM galactose + alpha lipoic acid, FA= food containing 555mM dextrose + 200mM galactose + folic acid, Pyr= food containing 555mM dextrose + 200mM galactose + pyroxidine, Thia= food containing 555mM dextrose + 200mM galactose + thiamine.

larvae grown on dex + gal food to see if any individual supplement significantly rescued survival to pupation or to adulthood (**Figure 10**). We applied a Chi-Square Analysis with 6 degrees of freedom to test significance of the effect. This analysis showed a significant effect of the five individual compounds on survival to both pupation (**Figure 10a**, p=0) and adulthood (**Figure 10b**, p=0).

**Table 4** presents all pairwise p-values obtained from post-hoc Chi-Square Analysis with a Bonferroni adjusted significance level of 0.002381. The striking result is that cobalamin alone accounted for the impact of the Nervous System Function group on survival. Indeed, cobalamin was the only compound that showed a significant positive effect on survival of galactose exposed GALT-null larvae to both pupation (p=0.00001068) and adulthood (p=9.60E-7). Addition of ALA significantly decreased survival to pupation (p=0.00058598), but not to adulthood (p=0.01091564). Addition of Thiamine, Folic Acid, nor Pyroxidine had no significant effect on acute galactose sensitivity to both pupation (p=0.92, p=0.11177103, p=0.22487296, respectively) and to adulthood (p=0.8265807, p=0.72, p=0.63078333, respectively) when compared to survival on dex + gal food. **Table 4** also shows the percent increase or decrease (positive or negative, respectively) of survival to adulthood of larvae exposed to each compound as compared to larvae eating dex + gal food alone.

| Comparison<br>Dex + Gal (n=100)<br>Dex + Gal + NS (n=120)<br>Dex + Gal + Cob (n=120)<br>Dex + Gal + Thia (n=120)<br>Dex + Gal + FA (n=120)<br>Dex + Gal + Pyr (n=120)<br>Dex+ Gal + ALA (n=120) | Increased vs. Decreased Survival to Adulthood Compared to Dex + Gal | % Increase or Decrease in Survival to Adulthood Compared to Dex + Gal | P-value Survival to Pupation | P-value Survival to Adulthood |
|---|---|---|---|---|
| Dex + Gal vs. Dex + Gal + NS | Increase | 30.50** | 0.0000054** | 0.00000557** |
| Dex + Gal vs. Dex + Gal + Cob | Increase | 33.00** | 0.00001068** | 9.60E-7** |
| Dex + Gal vs. Dex +Gal + Thia | No Effect | 1.33 | 9.20E-1 | 0.8265807 |
| Dex + Gal vs. Dex +Gal + FA | No Effect | 2.17 | 0.11177103 | 7.22E-01 |
| Dex + Gal vs. Dex +Gal + Pyr | No Effect | -2.83 | 0.22487296 | 0.63078333 |
| Dex + Gal vs. Dex +Gal + ALA | No Effect | -13.33 | 0.00058598** | 0.01091564 |
| Dex + Gal + NS vs. Dex + Gal + Cob | -- | -- | 0.87190032 | 6.94E-1 |
| Dex + Gal + NS vs Dex +Gal + | -- | -- | 0.0000037** | 0.00000501** |
| Dex + Gal + NS vs. Gal + FA | -- | -- | 0.00178253** | 0.00000947** |
| Dex + Gal + NS vs. Gal + Pyr | -- | -- | 0.00045199** | 1.50E-7** |
| Dex + Gal + NS vs. Gal + ALA | -- | -- | 0** | 0** |
| Dex + Gal + Cob vs Dex +Gal + | -- | -- | 0.00000752** | 7.80E-7** |
| Dex + Gal + Cob vs. Gal + FA | -- | -- | 0.00297783 | 0.00000155** |
| Dex + Gal + Cob vs. Gal + Pyr | -- | -- | 7.95E-4** | 2.00E-8** |
| Dex + Gal + Cob vs. Gal + ALA | -- | -- | 0** | 0** |
| Gal + Thia vs. Dex +Gal + FA | -- | -- | 0.11805026 | 0.88753708 |
| Gal + Thia vs. Dex +Gal + Pyr | -- | -- | 0.24250033 | 0.46326278 |
| Gal + Thia vs. Dex +Gal + ALA | -- | -- | 0.00022486** | 0.00422382 |
| Gal + FA vs. Dex +Gal + Pyr | -- | -- | 0.6919337 | 0.38114673 |
| Gal + FA vs. Dex +Gal + ALA | -- | -- | 2.10E-7** | 0.00271758 |
| Gal + Pyr vs. Dex +Gal + ALA | -- | -- | 0.00000148** | 0.03156441 |

**Table 4.** Pairwise comparisons between all the groups shown in **Figure 10**. Column 1 states the groups being compared. Column 2 states the survival effect of the individual compound on galactose exposed larvae. Column 3 reports the percent increase or decrease of larvae survival to adulthood in the presence vs. absence of the additives. Columns 4 and 5 report p-values of comparisons of survival to the pupal and adult stages, respectively. All p-values were calculated via post-hoc Chi-Square Analysis, α=.002381. **indicates statistical significance. Abbreviations explained in **Figure 10** legend.

**Timing of Galactose Toxicity:**

In an attempt to identify the stage at which vulnerability to galactose toxicity occurs, we measured the percent survival change between crucial stages in *Drosophila* development for larvae in each experiment. **Table 5** shows the percent losses from larvae to pupae, pupae to adults, and total loss from larvae to adults for cohorts of larvae deposited on dex + gal food and larvae deposited on dex + gal containing "mito-cocktail." It appears that more death occurred before pupation in the absence of the "mito-cocktail," however similar amounts of death occurred between pupation and adulthood in both the presence and absence of the "mito-cocktail."

| Condition<br>Dex + Gal (n=473)<br>Dex + Gal + NS (n=460) | % Loss from L1 to Pupae | % Loss from Pupae to Adults | % Total Loss from L1 to Adults |
|---|---|---|---|
| Dex+ 200mM Gal | 64.90 | 22.41 | 87.32 |
| Dex+ 200mM Gal + MM | 39.35 | 21.52 | 60.87 |

**Table 5.** Percent losses between *Drosophila* developmental stages on dex + gal food vs. dex + gal food plus the "mito-cocktail."

**Table 6** shows the percent losses from larvae to pupae, pupae to adults, and larvae to adults for GALT-null *Drosophila* exposed to each functional group of additives. It appears that more death occurred before pupation, in the absence of the

| Condition<br>Dex + Gal (n=480)<br>Dex + Gal + IS (n=300)<br>Dex + Gal + V (n=292)<br>Dex + Gal + ATP (n=300)<br>Dex + Gal + NS (n=280)<br>Dex + Gal + AO (n=280) | % Loss from L1 to Pupae | % Loss from Pupae to Adults | % Total Loss from L1 to Adults |
|---|---|---|---|
| Dex +200mM Gal | 51.46 | 21.25 | 72.71 |
| Dex +200mM Gal + IS | 38.00 | 28.67 | 66.67 |
| Dex +200mM Gal + V | 52.74 | 33.40 | 86.14 |
| Dex +200mM Gal + ATP | 70.00 | 14.00 | 84.00 |
| Dex +200mM Gal + NS | 23.57 | 30.00 | 53.57 |
| Dex +200mM Gal + AO | 49.93 | 25.00 | 73.93 |

**Table 6.** Percent losses between *Drosophila* developmental stages on dex + gal food and dex + gal food plus additives for the different functional listed groupings in Table 2.

Nervous System Function or Immune System Function groups. However, similar amounts of death occurred between pupation and adulthood occurred in both the presence and absence of a functional group. Overall, the Nervous System Function group had the lowest total percent loss (53.57%).

**Table 7** shows the percent losses from larvae to pupae, pupae to adults, and total loss from larvae to adults from cohorts of larvae deposited on dex + gal food and larvae deposited on dex + gal containing individual compounds from the Nervous System Function group. It appears that less death occurred before pupation in the presence of the cobalamin (20.83%), however similar amounts of death occurred between pupation and adulthood in both the presence and absence of any additional individual compound.

| Condition<br>Dex + Gal (n=100)<br>Dex + Gal + NS (n=120)<br>Dex + Gal + Cob (n=120)<br>Dex + Gal + Thia (n=120)<br>Dex + Gal + FA (n=120)<br>Dex + Gal + Pyr (n=120)<br>Dex + Gal + ALA (n=120) | % Loss from L1 to Pupae | % Loss from Pupae to Adults | % Total Loss from L1 to Adults |
|---|---|---|---|
| Dex + 200mM Gal | 49.00 | 24.00 | 73.00 |
| Dex + 200mM Gal + NS | 20.00 | 22.50 | 42.50 |
| Dex + 200mM Gal + Cob | 20.83 | 19.17 | 40.00 |
| Dex + 200mM Gal + Thia | 48.33 | 23.33 | 71.67 |
| Dex + 200mM Gal + FA | 38.33 | 32.50 | 70.83 |
| Dex + 200mM Gal + Pyr | 40.83 | 35.00 | 75.83 |
| Dex + 200mM Gal + ALA | 71.67 | 15.00 | 86.67 |

**Table 7.** Percent losses between *Drosophila* developmental stages on dex + gal food and dex + gal food plus the individual compounds in the Nervous System Function group.

**Long -Term Outcomes:**

We also conducted a small pilot study the effects of the full “mito-cocktail” and the Nervous System Function group on the climbing abilities of GALT-null *Drosophila*. In this pilot,

neither addition of the full “mito-cocktail” nor the addition of the Nervous System Function group to molasses- based fly food had a significant impact (p=.538672) on the climbing abilities of GALT-null *Drosophila* (**Figure 11**). We analyzed the data in **Figure 11** using Chi- Squared analysis with two degrees of freedom and applied a significance threshhold of 0.05. We found that neither the addition of the “mito-cocktail” nor the addition of the Nervous System Function group to molasses-based fly food significantly improved climbing ability of GALT-null *Drosophila* compared to GALT-null *Drosophila* reared on molasses-based food without additives.

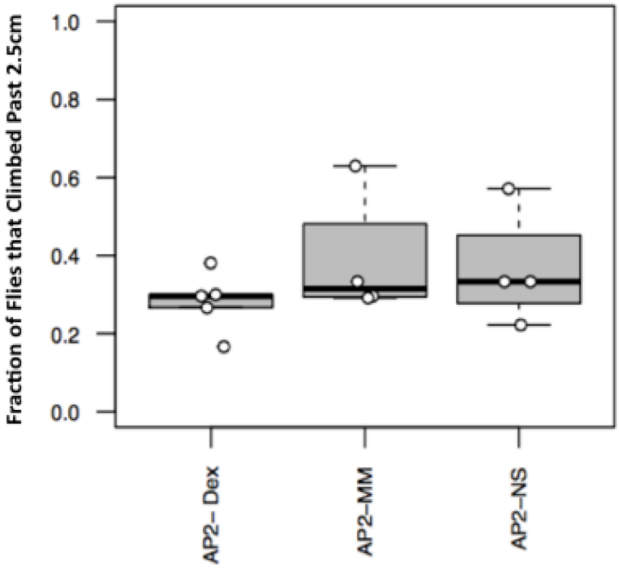


**Figure 11:** Climbing proportion *GALT -/- Drosophila* (AP2) raised on molasses-based food with or without “mito-cocktail” or the Nervous System Function group (p=0.14949385). Dex=control molasses-based food, MM= control molasses-based food + ”mito-cocktail,” NS= control molasses-based food + Nervous System Function group.

**DISCUSSION:**

In this study, we set out to determine if dietary supplements used to treat patients with mitochondrial defects (**Table 1)** might have an effect on acute galactose sensitivity and/or long-term outcomes of GALT-null *Drosophila melanogaster*. We also investigated whether certain functional groupings of a full "mito-cocktail," shown in **Table 2**, have a comparable effect to that of the full "mito-cocktail" on both acute galactose sensitivity and long-term outcomes of GALT-null *Drosophila*. Further, of the functional groupings that had a positive effect, we investigated whether individual components of the groupings had a positive effect on acute galactose sensitivity on their own. Lastly, we attempted to identify the stage at which vulnerability to galactose toxicity occurs in galactose exposed GALT-null *Drosophila*.

**Acute Outcomes:**

Our data present multiple novel findings regarding galactose sensitivity in GALT-null *Drosophila*. First, we found that addition of our "mito-cockail" to galactose-exposed GALT-null larvae improved survival at the tested concentration to both pupation ($p<0.00001$) and adulthood ($p<0.00001$). We can conclude that the addition of our "mito-cocktail" to dex + gal fly food showed a significant positive effect, minimizing acute galactose sensitivity of GALT-null *Drosophila*. Second, when examining various functional groupings of our "mito-cockail," we found that the Nervous System Function group showed a significant positive effect, minimizing acute galactose sensitivity of GALT-null larvae to both pupation ($p=0$) and adulthood ($p=8.00E-08$) when compared with survival on dex + gal food alone. Therefore, we may also conclude that the addition of the Nervous System Function group to dex + gal fly food showed a

significant positive effect on acute galactose sensitivity of GALT-null *Drosophila*. The simplest conclusion is that the components of the "mito-cocktail" in the Nervous System Function group—ALA, Cobalamin, Folic Acid, Pyridoxine, and Thiamine—were responsible for the impact seen with the original "mito-cocktail." Finally, we found that addition of cobalamin alone to dex + gal fly food fully accounted for the partial rescue of survival of galactose-exposed GALT-null larvae to both pupation ($p=0.00001068$) and adulthood ($p=9.60E-7$) when compared with survival on dex + gal fly food alone. As above, the simplest conclusion is that only one of the nine components of the original "mito-cocktail"—cobalamin—contributed to a significant positive effect on survival of galactose exposed GALT-null *Drosophila*. However, our sample size for the experiment examining cobalamin alone was small ($n=120$) compared to that of the other experiments ($n\approx400$), so it should be repeated.

**Timing of Galactose Toxicity:**

We also attempted to identify the stage at which vulnerability to galactose toxicity occurred in each experiment by measuring the percent losses between crucial stages in *Drosophila* development. As a preliminary conclusion, we found that GALT-null *Drosophila* were most vulnerable to galactose toxicity when developing from larvae to pupae. **Tables 5, 6,** and **7**, show that in the absence of any food additives, galactose exposed GALT-null *Drosophila* experienced more death when developing from larvae to pupae, than from pupae to adults. **Tables 5, 6,** and **7** show that addition of the full "mito-cocktail," the Nervous System Function group, and cobalamin, respectively, decreased the percentage of death from larvae to pupae. However, these patterns are anecdotal and must be tested further to be conclusive.

**Long-Term Outcomes:**

Our study also aimed to examine if the addition of a "mito-cocktail" to molasses-based fly food had a significant effect on the climbing abilities of GALT-null *Drosophila*. We found that neither the addition of the "mito-cocktail" nor the addition of the Nervous System Function group to molasses-based fly food significantly improved climbing activity of GALT-null *Drosophila* compared to counterparts climbing on unsupplemented molasses-based fly food. However, we had very limited statistical power for this experiment due to difficulties raising *Drosophila* on supplemented food at 28° Celsius. Therefore, we cannot definitively conclude that supplementation of molasses-based fly food with the "mito-cocktail" or Nervous System Function Group does not affect climbing performance.

**Further Observations and Limitations:**

We also made a number of qualitative or anecdotal observations while performing our experiments that may be worth noting. For example, when determining if the "mito-cocktail" affected acute galactose sensitivity, we noted that both C2 and AP2 *Drosophila* raised on dextrose food supplemented with the "mito-cocktail" developed faster by about 24 hours compared to those developing on unsupplemented dextrose food. However, when the "mito-cocktail" was added to dex + gal food, we did not witness a change in developmental speed of the animals.

Since previous studies[5] found that addition of antioxidants such as Vitamin C and α-mangostin had a positive impact on acute galactose sensitivity, we were perplexed to see that

our proposed Antioxidant functional group showed no positive effect. We also noted that both of the functional groups that contained L-carnitine and CoQ10, ATP Production and Antioxidant, either decreased or had no effect on survival. Perhaps L-carnitine and CoQ10 inhibit the actions of other compounds in these functional groups, or interact in a way that hinders a positive effect? Further work would be required to explore these possibilities.

As mentioned above, we also encountered a number of obstacles in conducting tests of long-term outcomes, specifically raising *Drosophila* at 28**°** on supplemented food. We first made homemade food following the protocol used for survival assay food. When allowing *Drosophila* to mate and lay embryos on this food for 48 hours and when loading L1 larvae onto this food, virtually no animals developed. When melting molasses-based fly food and supplementing as described in the Methods section and allowing *Drosophila* to mate and lay embryos on this food for 48 hours, again, virtually no animals developed. Only when L1 larvae were loaded onto supplemented melted molasses-based fly food, as described in the Methods section, did animals develop, however numbers were small, which is why we had low statistical power. The higher temperature of 28**°** Celsius may stresses the animals and might render them more vulnerable to something that they are not vulnerable to at 25**°** Celsius, alternatively, perhaps some of the compounds in the "mito-cocktail" act differently at 28**°** Celsius and have a negative effect on animal development.

**Future Directions:**

While our data are promising, more of these experiments are needed. Specifically, we need to achieve more statistical power for the comparison of the individual components in the

Nervous System Function group. Our conclusions regarding the affects of our “mito-cocktail” and certain subsets/components of it on acute galactose sensitivity are promising, thus we cannot discount that it may positively affect long-term outcomes as well. Our difficulties in executing this experiment and low statistical power limited us from making a definitive conclusion, thus this must be further investigated. Finally, we do not know that the dosages of compounds used here were optimal. Therefore, it is possible that dose-response titration experiments would reveal impacts we did not observe.

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## APPENDICES

| Abbreviation | Definition |
|---|---|
| GALT-null | Lacking the enzyme activity of GALT |
| *GALT +/+ and GALT -/-* | An animal with or without the gene GALT |
| Dex | Dextrose |
| Gal | Galactose |
| Dex + Gal | Dextrose food supplemented with 200mM galactose |
| C2 | Wild-type *Drosophila* |
| AP2 | GALT-null *Drosophila* |
| MM | "Mito-cocktail" or "mito-mix" |
| NS | Nervous System |
| IS | Immune System |
| AO | Antioxidant |
| Vit | Vitamin digestion/ absorption |
| Cob | Cobalamin |
| Thia | Thiamine |
| FA | Folic Acid |
| ALA | Alpha Lipoic Acid |
| Pyr | Pyroxidine |
| CoQ10 | Coenzyme Q10 |

**Appendix 1:** Definitions of common abbreviations found in the paper.