Diagnosis and prevention of metabolic diseases in Drosophila melanogaster

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Diagnosis and prevention of metabolic diseases in *Drosophila melanogaster*  
Claire Coogan  
Engaged Learning 2013 - Final Project  
Mentor: Dr. Johannes Bauer
Section 1. Introduction

*Drosophila melanogaster* has an expansive and impressive history as a model organism. From its central role in three Nobel prize winning studies to its modern day usage in cutting-edge research related to aging and Alzheimer’s [1], the *Drosophila* model has proven itself to be one of the greatest assets to science and medicine. The often-used murine model for metabolic research suffers from high maintenance costs and lengthy developmental time; *D. melanogaster* makes a generally more efficient model because of the fruit flies’ conveniently short life cycle and inexpensive upkeep. Additionally, *Drosophila* is superior at performing genetic screens, which is particularly useful when researching metabolism, which has many genetic influences.

There is a high degree of conservation among the neuroendocrine and metabolic architecture of members of the animal kingdom. The universal necessity to maintain blood glucose homeostasis means that the insulin signaling pathway and the storage of excess glucose in the form of glycogen and triacylglycerides are some of these evolutionarily ancient and highly conserved pathways. Thus our understanding of human metabolic diseases can be productively advanced through use of animals that are much more distantly related to humans than mammals. *Drosophila* seems a potentially ideal model organism for this research.

One of the most widespread metabolic diseases in the United States of America is type II diabetes mellitus. The majority of Americans with diabetes have been diagnosed with type II and the number affected grows every year since risk factors include advanced age and calorie-excessive diet. Despite its prevalence, not much is known about how to best treat this disease or other metabolic diseases. Modern anti-diabetic treatment and prevention options are suboptimal and vary in efficacy. Discovery of and research into new treatment options is essential to optimize treatment of the disease. The use of the proven model organism, *D. melanogaster*, in a drug screen will help to expedite these discoveries.

1.1 *Drosophila* in metabolic research

This endeavor is not without precedent. A number of studies have already been performed which employ *Drosophila* as a model for metabolic disease. There has been substantial research done which indicates mammalian insulin and *Drosophila* insulin-like peptides (dILPs) perform similar functions and participate in analogous pathways, including glucose storage regulation and certain aging pathways [2,3]. Further *Drosophila* research regarding the specifics of blood glucose homeostasis has yielded informative results [4]. This success has promoted interest in using *Drosophila* to study metabolic disorders that result in a person’s inability to autonomously maintain this homeostasis.
Of particular interest is the development of diet-induced metabolic disease. Insulin resistance is the inability of cells to respond to insulin and is the primary characteristic of type II diabetes mellitus. This insulin resistant phenotype (as well as obesity) was observed in flies fed high-sugar diets [5]. This result is consistent with those obtained by previous studies in our lab. In these studies it was shown that flies overfed on yeast or sugar experience weight gain and are observed to be insulin resistant [6]. These flies are similarly seen to have decreased longevity. Flies fed on 5% yeast or sucrose may be healthier than those fed on 30% yeast or sucrose - additional data also supports this idea. Insulin resistance and its effect can be assessed using a number of techniques. Of these techniques, one of the most easily quantifiable is the metabolic storage phenotype. Insulin resistance results in abnormal trends in glucose storage. To monitor these trends, the concentration of glucose, glycogen, and trehalose can be measured. The effect of diet on the glucose storage phenotype of Drosophila and results are summarized in figure 2 and figure 3. Note how dramatically different the “healthy” 5% fed flies are from the “unhealthy” 30% fed flies.

Figure 1. Effects of a high protein diet on Drosophila metabolism. Following eclosion, adult flies were raised for ten days on a diet of sucrose (15%, all contents w/V) and differing amounts of yeast extract as indicated. Flies were harvested, weighed and their glucose, glycogen and trehalose levels determined in whole body extracts in at least triplicate. Flies show weight gain with increased food content when on non-starvation diets (1-way ANOVA pb0.0001; with post-test for linear trend p=0.0023; asterisk; t-test for the weight drop between 15% and 30%; p=0.0038). Glycogen and trehalose levels decline with increasing food content (1-way ANOVA with post-test for linear trend: pb0.0001and p=0.0108, respectively), but no changes in glucose levels are observed. Shown is a representative of two independent experiments. Figure and legend taken directly from Morris et al [6].
The major implications of figures 2 and 3 are evident from examining only the effects of 5% (“healthy”) and 30% (“overfed”) diets. As the yeast extract content of their diet increases, fly mass increases, total glucose levels stay constant, while glycogen and trehalose levels decrease. As the sucrose content of their diet increases, fly mass increases, while glucose, glycogen, and trehalose levels stay essentially constant. These trends will serve as the baseline for assessing drug effect on the flies.

The initial drug screen therefore is limited to the two pairs of 5% and 30% yeast and sucrose foods to optimize efficiency.

1.2 My project goals: Finding novel anti-diabetic treatments

The goal of this study is to use this previously published data as a baseline to assess the efficacy of commonly prescribed human anti-diabetic medications on Drosophila. The known anti-diabetic compounds that I will use on the flies are glibenclamide and rosiglitizone. Ria Chaabra, a high school student involved in the lab this summer, had a personal interest in traditional Indian medicine. She chose to investigate the effect of different natural compounds in her own naturalistically inspired “drug” screen following...
my design of experiments. Her summer data yielded promising results that prompted me to add *Cinnamomum cassia* to my Engaged Learning drug screen.

It is important to note that medication can be taken to do either of two things: prevent the development of unhealthful phenotypes or treat the disease after it has already developed. Note that this screen is designed to assess the preventative effects of these medications and compounds, not their impact as part of a treatment regimen. Theoretically, an effective prevention measure should result in halted development of the unhealthy phenotype in flies fed unhealthy food. In other words, application of an effective prevention medication should result in a reversion of the “unhealthy” 30% fed phenotype to be more like that of the “healthier” 5% fed flies.

Preliminary data has been obtained for the known anti-diabetic medication metformin and the naturally derived compound, curcumin. Curcumin is a component of turmeric, a spice considered healthful in traditional Indian medicine. The preliminary mass data obtained for curcumin and metformin is summarized in figure 4.

![Figure 3: Effect of (a) metformin and (b) curcumin on the mass of 5% and 30% yeast extract diet *Drosophila*. Drug concentration in food increases as color saturation increases: white is the control vehicle, the light color is [A], the mid-tine is [B], and the most saturated color is [C]. Note that this data is very preliminary: quantities and error bars shown are the result of single experiments where samples were obtained in biological quadruplet. Data in (b) was collected independently by Ria Chaabra. All data shown obtained for female 10 day old flies. Data for sucrose not shown.](image)

In examining results of the drug screen we look for evidence of a phenotype reversion. If a compound or medication has a preventative effect on the development of the unhealthy phenotype seen in the control 30% yeast extract, then the flies fed the drug or compound treated foods in the 30% food should show a dose response curve that trends towards resembling the healthier, 5% yeast extract fed control flies. Note that such a reversion is not indicated by this preliminary data for either of these additives. The data for both metformin and curcumin indicate that the compounds may show potentially drug concentration dependent response curves.
The curves seem to indicate a general decreasing trend in mass as more metformin is added, and a generally increasing trend in mass as more curcumin is added to the food. However, the effect of either additive is not indicative of a reversion to a healthy phenotype and is observable in both the healthy and overfed flies. Thus, preliminary results of the screen indicate that these drugs do not seem to prevent the development of the unhealthy mass phenotype exhibited by protein overfed *Drosophila.*
Section 2. Materials and Methods

2.1 Fly background
The line tGPH was a kind gift from B. Edgar (Fred Hutchinson Cancer Research center, Seattle, WA). This line expresses a GFP-PH domain in a wildtype w¹¹8 background. This line is the same line used to obtain previously published data on insulin resistance and metabolite quantification [6].

2.2 Diet
2.2.1 Stock diet
Regular food used for stock maintenance contained approximately 11% sucrose, 3% yeast, and 5% corn meal by mass. This regular food recipe is a commonly used recipe that is composed to maximize stock productivity and hardiness.

Fly stocks were raised on approximately 10-15mL regular food topped with a small amount of activated yeast to encourage stock vitality and virility. All experimental flies consumed this regular food while developing as larvae but were collected and transferred to experimental food conditions within 24 hours of eclosion.

2.2.2 Experimental diet
Experimental food was prepared with varying yeast extract and sucrose content as described previously [6]. Four of these food conditions were employed in this drug screen: 5% and 30% yeast extract with sucrose constant at 15%, as well as 5% and 30% sucrose content with yeast extract constant at 15%. Medications and compounds employed in this drug screen were added to these four kinds of food.

2.3 Medications and natural compounds
The medications tested were glibenclamide, and rosiglitizone and the natural compound tested was Cinnamomum cassia, commonly known as cassia cinnamon. Preliminary assays were also performed involving metformin and curcumin, the active ingredient in turmeric. Each medication or compound was added to fly food in 3 different concentrations: low [A], mid-level [B], and high [C]. Control vehicle (CV) food was also created for each drug to account for solvent effects. CV foods have no medication in them, only 20 mL solvent/L, the same concentration of solvent used in the [C] foods. This is done to avoid falsely attributing solvent effects to some action of the solvated drugs. Cinnamon was added as a powder and the cinnamon CV food accordingly contains no additional liquid.

The specific concentration for each drug is given in table 1. Justification for these concentrations is given in section 1.3.1
<table>
<thead>
<tr>
<th>Name of medication or compound</th>
<th>Recommended daily dose for human adult diabetic</th>
<th>Lowest concentration, [A]</th>
<th>Mid-level concentration, [B]</th>
<th>Highest concentration, [C]</th>
<th>Control vehicle identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin</td>
<td>500 mg</td>
<td>0.8 g / L food</td>
<td>1.6 g / L food</td>
<td>3.2 g / L food</td>
<td>H₂O</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>5 mg</td>
<td>8 mg / L food</td>
<td>16 mg / L food</td>
<td>32 mg / L food</td>
<td>CH₃CH₂OH</td>
</tr>
<tr>
<td>Rosiglitizone</td>
<td>2 mg</td>
<td>3.2 mg / L food</td>
<td>6.4 mg / L food</td>
<td>12.8 mg / L food</td>
<td>CH₃CH₂OH</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>Not known</td>
<td>1 g / L food</td>
<td>2 g / L food</td>
<td>4 g / L food</td>
<td>None</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Not known</td>
<td>0.5 g / L food</td>
<td>1 g / L food</td>
<td>2 g / L food</td>
<td>(CH₃)₂CO</td>
</tr>
</tbody>
</table>

**Table 1. Summary of experimental food conditions**

2.3.1 Calculation specifics:

Metformin daily recommended daily dose for a diabetic human is 500mg. The human medication is orally administered. Since flies live on the food they eat and we do not have technology to force feed them, this dose could not be directly scaled down based on fly mass. Preliminary experiments determined an effective food-distributed dose to be 1.6g metformin / L food.

To widen the range of conditions in the drug screen, this preliminary dose was halved and doubled to give additional ([A] and [C]) metformin concentrations of 0.8g/L and 3.2g/L. Based on this, calculations were done to determine the dose for the other medications and compounds. The results of these calculations are summarized in table 1, located in section 1.3.

2.4 Fly husbandry

All flies were raised in food-containing clear polystyrene vials topped with rayon plugs. Flies were kept in an incubator set to maintain 50% humidity and 25° C on a 12 hour light/dark cycle.

The fly density of stock vials was monitored carefully, lest the environment become detrimental to larval development and feeding. Stock vials were discarded before the food was approximately 4 weeks old as a preventative measure against the development of mites.

2.5 Metabolic assay

Flies were raised for ten days on experimental food conditions. On day 10 they were collected and weighed. Fly collection is discussed in detail in section 1.5.1. Samples were prepared by decapitating flies, pulverizing the bodies with a pestle in a hypotonic buffer.
solution, followed by processing the supernatant to quantify metabolites. Sample preparation is discussed in detail in section 1.5.2. Metabolite quantification is accomplished using Sigma kits, enzymes and use of absorption spectroscopy. The biochemistry of the kits is detailed in section 1.5.3.

2.5.1 Experimental conditions

Adult flies were collected under light CO$_2$ anesthesia from stock vials within 24 hours of eclosion and distributed in groups of 50 onto experimental food conditions. Each experimental vial contained 25 males and 25 females and approximately 5mL of the appropriate food. The flies were passed to vials containing new food approximately every 48 hours to ensure consistent food quality. The flies were aged for 10 days in this manner.

2.5.2 Collection and sample preparation

On day 10, flies were anesthetized using CO$_2$, separated by sex, and deposited in groups of 4 or 5 into preweighed eppendorf tubes. After mass was recorded, samples were labeled and stored at -80°C until further processing could take place. Note that after this point all samples are kept on ice or colder after collection to avoid decomposition or uneven enzyme activity.

Samples were flash-frozen in N$_2$(l) and then vortexed to remove heads from bodies and heads discarded. This is done because eye pigment may interfere with absorption readings. To finish preparing samples for spectrophotometric analysis of sugars, the metabolites must first be liberated from the fly body into a protective buffer solution. Buffer A was added to the bodies in the quantity of 100uL buffer per fly body. Samples were then ground-up and separated by centrifuge. The supernatant is the completed sample and was examined using the appropriate Sigma assay kit and enzymes.

Buffer A is often used in our lab for general purposes and so contains a number of compounds designed to protect proteins from degradation as well as reducing agents. The composition of buffer A is identical to that of the buffer used in previous lab metabolic studies [6].

2.5.3 Data collection – Sugar quantification

2.5.3.1 Glucose
To determine glucose levels, each sample is pipetted in 10uL triplicates into 96-well clear-bottom plates. After plating, reconstituted Sigma glucose assay reagent is added to each well and the plates are allowed to incubate for 15 minutes. After 15 minutes, the absorption is measured at 340nm using a plate reader. Data is processed by Gen5 software.

The sigma glucose assay reagent kit contains glucose-6-phosphate dehydrogenase (G-6-PDH), hexokinase, ATP and all necessary cofactors. The enzymes in the kit perform the following reactions:
These reactions allow glucose levels to be spectrophotometrically assessed by producing a proportional amount of NADPH whose concentration can be quantified by determination of absorbance at 340nm.

2.5.3.2 Glycogen

100μL aliquots of each sample are treated with amyloglucosidase and allowed to incubate for 45 minutes at 55°C. This allows the amyloglucosidase to perform the following reaction:

This creates a proportional amount of glucose from the glycogen present which allows for spectrophotometric quantification of the glycogen present using the sigma glucose assay reagent.

The amyloglucosidase treated sample is then processed as described in 1.5.3.1. After fitting to a standard curve, total glucose levels are determined, the value of glucose obtained for non-glycogen glucose is subtracted from the reading to obtain the signal that can be attributed to glycogen levels. The Gen5 software is programmed to do this quantification.

2.5.3.3 Trehalose
100μL aliquots of each sample are treated with trehalase and allowed to incubate for 45 minutes at room temperature. This allows the trehalase to perform the following reaction:

![Chemical structure of trehalase and glucose](image)

This reaction creates a proportional amount of glucose from the trehalose present which allows for spectrophotometric quantification of the trehalose present using the sigma glucose assay reagent.

The trehalase treated sample is then processed and analyzed as described above for the glycogen determination.
Section 3. Project results

3.1 Anti-diabetic medications

3.1.1 Glibenclamide

It is immediately apparent from this data that there is no phenotypic reversion from unhealthy to healthy phenotype in any of the four tested metabolically relevant parameters. The data does not indicate that glibenclamide has any effect on the diet induced metabolic disruption observed in Drosophila.

Another thing that may seem odd in this data is that the CV data trends are not the same as the trends observed in that data published by Morris et al [6]. This is likely because the CV food features a large volume of solvent – ethanol in the case of glibenclamide. This additional ethanol means that the CV food is of a different composition from the previously examined food and that the CV flies are not directly
analogous to the flies that served as the baseline for this study. This disparity is not surprising because ethanol contributes additional calories and volume to the fly food. The ethanol could also interact somehow with the other food components which could lead to a different final food composition and, thus, very different phenotypic effects.

Figure 5. Effect of glibenclamide on metabolism of 5% and 30% yeast extract fed Drosophila. Drug concentration in food increases as color saturation increases: white is the control vehicle, the light color is [A], the mid-tine is [B], and the most saturated color is [C]. All quantities and error bars shown are the result of an average of at least three independent experiments where samples were obtained in biological quadruplet. Extracts for b, c, and d were plated in triplicate. All data shown obtained for female 10 day old flies.

The data from varying protein diet flies also does not indicate any phenotypic reversion. There may be a general, diet independent increasing trend in mass and glycogen content seen in both the health and overfed flies and statistics need to be performed to assess this.

The general metabolic trends seen in the CV food for the 5% and 30% yeast extract flies differs slight from what was observed in foods not containing ethanol by Morris et al. The only difference is that the glucose content seems to decrease from 5% to 30% rather than remaining constant. Again, differences in phenotype of the CV from
previous data is not surprising due to the effects arising from the high ethanol content of the CV food.

3.1.2 Rosiglitizone

Figure 6. Effect of rosiglitizone on metabolism of 5% and 30% sucrose fed Drosophila. Drug concentration in food increases as color saturation increases: white is the control vehicle, the light color is [A], the mid-tine is [B], and the most saturated color is [C]. All quantities and error bars shown are the result of an average of at least three independent experiments where samples were obtained in biological quadruplet. Extracts for b, c, and d were plated in triplicate. All data shown obtained for female 10 day old flies.

The data indicates no apparent drug effects.

Note again that the deviation of the CV trends from those anticipated from the baseline experiments are not surprising due to the ethanol content of the CV food.
Figure 7. Effect of rosiglitazone on metabolism of 5% and 30% yeast extract fed Drosophila. Drug concentration in food increases as color saturation increases: white is the control vehicle, the light color is [A], the mid-tone is [B], and the most saturated color is [C]. All quantities and error bars shown are the result of an average of at least three independent experiments where samples were obtained in biological quadruplet. Extracts for b, c, and d were plated in triplicate. All data shown obtained for female 10 day old flies.

There may be a rosiglitazone induced increase in glucose content seen in b, but it is not statistically relevant, nor diet dependent.

This data also does not indicate any statistically relevant drug responsive effects.

3.2 Natural compounds

3.2.1 Cinnamomum cassia
Figure 8. Effect of *C. cassia* on metabolism of 5% and 30% sucrose fed *Drosophila*. Drug concentration in food increases as color saturation increases: white is the control vehicle, the light color is [A], the mid-tone is [B], and the most saturated color is [C]. All quantities and error bars shown are the result of an average of at least three independent experiments where samples were obtained in biological quadruplet. Extracts for b, c, and d were plated in triplicate. All data shown obtained for female 10 day old flies.

The data in figure 8a indicates a mass phenotype reversion. The other metabolic phenotypes do not indicate any compound dependent effects at all.

Interestingly, the dose response curve does not show the strongest preventative result at the highest concentration of *C. cassia*, but rather at the lowest, [A]. This may indicate that the doses tested in this drug screen were in excess of the effective dose and that *C. cassia* is most beneficial in small amounts.
Figure 9. Effect of *C. cassia* on metabolism of 5% and 30% yeast extract fed *Drosophila*. Drug concentration in food increases as color saturation increases: white is the control vehicle, the light color is [A], the mid-tine is [B], and the most saturated color is [C]. All quantities and error bars shown are the result of an average of at least three independent experiments where samples were obtained in biological quadruplet. Extracts for b, c, and d were plated in triplicate. All data shown obtained for female 10 day old flies.

Figure 9a indicates a similar reversion in mass phenotype as is observed in figure 8a. The other metabolic parameters again seem unaffected by addition of the cinnamon. Again, the most effective concentration of cinnamon is the lowest, [A]. These data are nicely consistent with the data seen in figure 8 and implicate *C. cassia* as a potential preventative dietary additive to stave off development of an overfed mass phenotype.

**Section 4. Discussion**

No statistically relevant phenotype reversion was observed with addition of either of the known human anti-diabetic medications glibenclamide and rosiglitazone. Conversely, the data obtained for the natural compound, *C. cassia*, indicates that it has preventative effects on the development of increased mass in overfed *Drosophila*. This perhaps surprising result could be due to a number of reasons – all speculative until further tests are done.
It is possible that the parameters selected for this first drug screen are not sufficient to observe the action of these drugs in metabolic processes. Fertility assays could also be added to the screen to provide an additional parameter to further assess the comparative health of the medicated flies. Alternatively, insulin resistance could be directly monitored. This can be done by performing assays such as PH-domain membrane co-localization assays, *Drosophila* glucose tolerance tests, or western blots to quantify the proportion of phosphorylated to unphosphorylated Akt. However, these assays are not yet straightforward to perform in the lab and are currently time-consuming, low yield and thus ill-suited for a expansive, wide-scope initial screen such as this.

It is also possible that the pathway that results in *C. cassia* affiliated weight-gain prevention is more evolutionarily old than pathways associated with modern anti-diabetics. If this is true, then is it possible that human drugs won’t affect flies in the same way that they affect humans since the pathway only recently evolved.

It is possible that human anti-diabetics are not intended to prevent the development of an unhealthy phenotype with poor diet but only to aid in treatment of it, perhaps in conjunction with the adoption of a healthy diet. The assays performed in this drug screen all focused on prevention. Another drug screen could be performed where the flies are raised on healthy and overfeeding food conditions and are then periodically treated with the drugs and compounds. Results of such a screen would likely be very different from the results of this prevention-focused drug screen.

Another assay that would be informative is a feeding assay. A feeding assay quantifies the volume of each food eaten by flies. This assay could help to quantify exactly how much of each drug or compound each fly ingests which can aid in determining a mass proportional dose for further testing. This assay would additionally ascertain whether the observed loss in mass associated with *C. cassia* is due to some biological effect of the compound or simply due to the flies eating less due to the strong taste of *C. cassia* in the fly food. However, this result is unlikely because the strongest preventative effect observed was in the [A] cinnamon food. The [A] food contains cinnamon in an amount 1/4\textsuperscript{th} as concentrated as the [C] food and, even if they do dislike the taste of cinnamon and that affects their appetites, it does not make sense that the flies would eat less of the [A] food than the [C] food.

An interesting topic to pursue next is the origin of the weight-gain preventative effect of *C. cassia*. The levels of measured metabolites remain essentially constant across cinnamon concentrations so the phenotype reversion is not due to a loss in glucose, glycogen or trehalose. This weight loss could be due to a decrease in any number of other chemicals – ranging from triacylglycerides, to protein, to water. Further tests must be done to determine the nature of the observed weight loss.
Section 6. Conclusion
The results of the drug screen indicate *Cinnamonium cassia* as the most effective of the three food-additives tested. Cassia cinnamon prevents the development of unhealthy/excessive diet-induced weight gain. The cinnamon had a significant preventative effect on the one of the phenotypes measured, while the human anti-diabetic medications did not have a preventative effect on any of them. These results have helped to illustrate the potential usefulness of a *Drosophila*-based drug screen as a means to identify novel potential anti-diabetics or other potentially useful medications. The results of my Engaged Learning project can hopefully lead to the development of a more informative and efficient future *Drosophila* drug screen. The outcome of this project constitutes a positive contribution to the larger global health effort.
Section 7. References


