JOURNAL OF
EMERGING INVESTIGATORS

Article

Utilizing 25-Hydroxyvitamin D3 to prevent the appearance of diabetic-like phenotypes in *Drosophila melanogaster*

Ashni Zaverchand[1], Leya Jokutty[1], and Kepa Oyarbide[1]
[1]American Heritage School, Plantation, Florida

SUMMARY

Currently, over 415 million people worldwide have diabetes and within that population, 90–95% have type 2 diabetes; increasingly more children and young adults are also developing it. This study aimed to assess the role of 25-hydroxyvitamin D3 solution, at varying concentrations, in protecting vertical transmission of diabetic-like phenotypes. Fruit flies were suitable model organisms, as they share 75% of the disease-causing genes with humans and can develop diet-induced insulin resistance when reared on high-sucrose diets (HSDs). All fly groups (including parents and offspring) were utilized for assays including measuring adult body mass, wing size, and glucose and sugars content. We hypothesized that the highest concentration of vitamin D solution (55 ng/mL) would be most effective in having a protective role. The results indicated that the hypothesis was partially supported; overall, all three concentrations of the vitamin D solution administered to the flies reared on HSDs had a protective effect, to varying extents. Therefore, these results can be applied to show the role of 25-hydroxyvitamin D3 as a potential treatment for type 2 diabetes.

INTRODUCTION

Type 2 diabetes (T2D) is a chronic condition that results when the pancreas produces insulin, but the insulin produced is either inadequate or the body is unable to recognize it for proper use. Although T2D most often occurs in those over the age of 45, increasingly more children and young adults are also developing it, making it a prevalent issue that needs immediate attention (1).

A significant portion of vitamin D is obtained from the skin by UVB-induced conversion of 7-dehydrocholesterol to vitamin D3, followed by two hydroxylations that occur primarily in the liver and kidney, to produce 25-hydroxyvitamin D3 (25(OH)D) and 1,25-dihydroxyvitamin D3 (1,25(OH)2D) (2). A 12-year cohort study concluded that plasma 25(OH)D concentrations greater than 30 ng/mL were associated with lower hazard ratios for diabetes and 25(OH)D concentrations greater than 50 ng/mL were associated with even lower hazard ratios. Normal levels of 25(OH)D are between 20 ng/mL and 50 ng/mL. They found no association of 1,25(OH)2D with risk of T2D (3). There is evidence linking vitamin D deficiency to higher risks associated with the development of T2D. Metabolites of vitamin D have been shown to affect beta cell function and therefore, insulin secretion (4). Insulin secretion is a calcium-dependent process and vitamin D plays a regulatory role in the cytosolic calcium concentration and flux through the pancreatic beta cells. In vivo studies further support that vitamin D deficiency has been associated with impaired insulin secretion in rat pancreatic beta cells, while vitamin D supplementation helped to restore the impaired glucose-mediated insulin secretion in vitamin D-deficient rats (4). Vitamin D seems to have a direct effect, as 25(OH)D (the circulating active form) may bind to the vitamin D receptor expressed in beta cells, making this metabolite worthy of study (5).

Drosophila melanogaster, or fruit flies, can be used to model T2D in humans, as they can develop diet-induced insulin resistance and have the orthologous gene for the vitamin D receptor in humans, DHR96. Relevant findings from studies show that rearing Drosophila larvae on HSDs (HSDs) induces diabetic-like phenotypes such as decreased larval size and delayed larval development (6). Additionally, HSDs result in other observable diabetic-like phenotypes including reduced body weight and wing size (7).

Along with diet and lifestyle, genetics, as well as epigenetics, play a role in the pathogenesis of T2D (8). Epigenetics is the study of heritable changes to DNA, rather than the sequence. These changes may affect future generations and can cause changes in phenotype. Studies describing the role of DNA methylation (an epigenetic mechanism) in the etiology of T2D indicate that the majority of T2D risk genes showed increased DNA methylation, which negatively affected beta cells' function and therefore, insulin secretion (9). Recent studies indicate that Vitamin D may have a protective effect on the epigenome, by increasing the activity of DNA demethylases to counteract the inactivation of many diabetes-related genes due to hypermethylation (10).

Furthermore, a study that described the temporal requirements for insulin signaling during development in Drosophila indicates a temperature-sensitive insulin receptor (Inr) mutation in the flies that results when the mutant flies are shifted in growth from 18˚C to 24˚C at certain points in development (11). However, why these temperature-sensitive flies exhibit different phenotypes such as decreased size result, is unknown. Nevertheless, these flies served as viable (able to survive until adulthood) T2D models. However, these InR mutant flies differ in that they were born with some form of

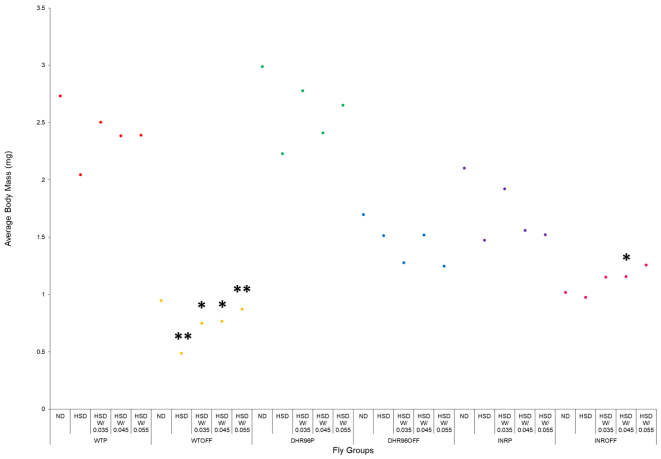

Figure 1: Wildtype offspring fed HSDs weighed significantly less than those that were supplemented with 25(OH)D. The adult body mass assay was performed with an average of forty-five flies per fly group tested. Statistically significant t-test results were obtained for the wildtype offspring comparing the HSD (WTOFFHSD) to that with varying concentrations of the vitamin D solution (WTOFFHSD W/ 0.035, WTOFFHSD W/ 0.045, WTOFFHSDW/ 0.055) (0.035 µL/mL, *p < 0.05; 0.045 µL/mL, *p < 0.05; 0.055 µL/mL, p < **0.01).

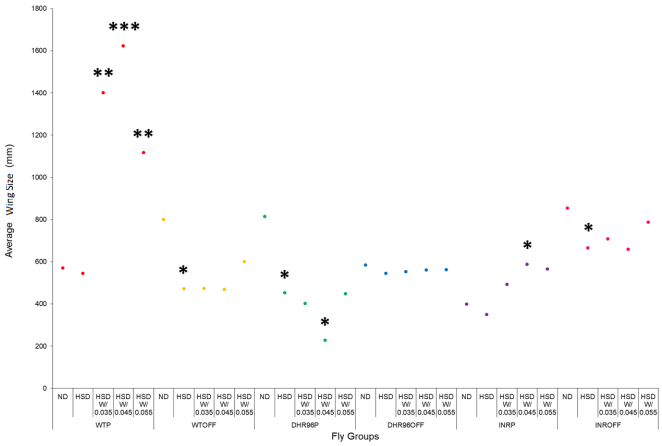

Figure 2: Wildtype parent flies fed HSDs experienced greater wing size reduction and damage than did fly groups supplemented with 25(OH)D. Fly wings were dissected, placed carefully on grid paper, and analyzed using ImageJ software (average of thirty flies). Statistically significant t-test results were obtained for the wildtype parental generation flies comparing the HSD (WTPHSD) to that with varying concentrations of the vitamin D solution (WTPHSD W/ 0.035, WTPHSD W/ 0.045, WTPHSD W/ 0.055) (0.035 µL/mL, *p < 0.05; 0.045 µL/mL, ***p < 0.001; 0.055 µL/mL, *p < 0.05).

the mutations causing diabetic-like phenotypes in comparison to the wildtype model that was reared on HSDs to induce diabetic-like phenotypes.

We wanted to determine and evaluate the extent to which 25(OH)D solution affects the diabetic-like phenotypes (reduced body mass, reduced wing size, and elevated sugar levels) in wildtype and InR mutant fruit flies. We conducted the same experiments with DHR96 mutant fruit flies to serve as a negative control and validate the results. As such, we predicted that when wildtype and InR mutant fly groups are reared on HSDs infused with 0.035 µL/mL, 0.045 µL/mL, or 0.055 µL/mL of 25(OH)D solution, then the offspring of only the flies given 0.055 µL/mL solution will not exhibit decreased body mass, decreased wing size, or elevated glucose, sucrose, or trehalose content. These concentrations are equivalent to 35 ng/mL, 45 ng/mL, and 55 ng/mL, respectively, in the human system. The concentration of the stock solution used for experimentation was 100 µg/mL. If shown to be effective in playing a protective role, 25-hydroxyvitamin D3 could have vast applications in the field of medicine as comparative analyses comparing this metabolite to current treatments to alleviate symptoms of T2D could be conducted. Additionally, understanding this metabolite of Vitamin D and its role in T2D could give insight into its possible effects in related diseases.

RESULTS

This study investigated the role of 25(OH)D solution in preventing the appearance of diabetic-like phenotypes in the offspring of the fruit flies. After rearing the fly groups on their corresponding diets, we measured the effects of the vitamin D solution by assessing adult body mass, wing size, and glucose, sucrose, and trehalose content in the flies. We hypothesized that the highest concentration of the 25(OH)D solution (0.055 µL/mL) would be most effective in preventing the appearance of diabetic-like phenotypes such as reduced adult body mass, wing size, elevated glucose, sucrose, and trehalose content in the wildtype and InR mutant flies. We expected that the DHR96 mutant flies would be unresponsive to the vitamin D solution, regardless of the concentration at which it was administered because they lack the vitamin D receptor; these flies served as the negative control in the experiment. The results from the DHR96 mutant fly groups helped to further validate the results seen in the offspring of the wildtype and InR mutant fly groups. An average of 45 flies were utilized for the adult body mass assay, 30 flies for that of the adult wing size/area assay, and 10 flies (each) for the assay measuring the amounts of glucose, sucrose, and trehalose in the fruit flies.

Reduced body mass was a diabetic-like phenotype that was measured in this study. As per the results from the adult body mass assay, we found that the highest concentration of the vitamin D solution (0.055 µL/mL) yielded similar body masses for the offspring of wildtype and InR mutants — values closest to that seen in the flies reared on normal diets. The vitamin D solution, at all three concentrations (0.035 µL/mL, 0.045 µL/mL, and 0.055 µL/mL) had somewhat of a protective effect on the offspring of the wildtype and InR mutant fruit flies, but was most effective at the highest concentration administered, even though only a slight improvement was noted amongst the three concentrations (Figure 1). We performed t-tests to evaluate our experimental and control groups. Wildtype offspring fed HSDs were significantly smaller than those that were supplemented with 25(OH)D

(0.035 µL/mL, $p < 0.05$; 0.045 µL/mL, $p < 0.05$; 0.055 µL/mL, $p < 0.01$).

Decreased wing size is an indicator of diabetes in fruit flies fed HSDs. The objective of this assay was to simply measure and compare the wing size of the flies. In the wildtype parental generation, the concentration of 0.045 µl/mL of vitamin D solution was the most effective. On the other hand, in the InR mutant parental generation, the lowest concentration (0.035 µl/mL) was effective in yielding similar wing sizes to the flies reared on normal diets. (Figure 2). Wildtype parent flies fed HSDs experienced greater wing size reduction and damage than did fly groups supplemented with 25(OH)D (0.035 µL/mL, $p < 0.05$; 0.045 µL/mL, $p < 0.001$; 0.055 µL/mL, $p < 0.05$). Other statistically significant results were obtained within the wildtype and InR mutant offspring, DHR96 mutant, and InR mutant parental generation. The three concentrations of the vitamin D solution administered to the flies reared on HSDs were effective in protecting the wings to some extent; there was still a reduction in wing size but not to the extent to which those only fed HSDs was observed. There was a statistically significant protective effect in the wildtype parental generation overall.

The glucose results indicated that at lower concentrations of the vitamin D solution, glucose content was higher than that of the flies reared on normal diets (Figure 3). This was also observed with the sucrose content (Figure 4) and trehalose content (Figure 5) results. However, the sugar contents overall were similar between the wildtype and InR mutant offspring fly groups reared on normal diets and those reared on HSDs with the highest concentration of the vitamin D solution. Wildtype parent flies fed HSDs had greater glucose content than did fly groups supplemented with 25(OH)D (0.035 µL/mL, $p < 0.0001$; 0.045 µL/mL, $p < 0.0001$; 0.055 µL/mL, $p < 0.0001$). Within the sucrose and trehalose content assays, other statistically significant results were obtained for certain group comparisons; however, once again, as with the wing size assay, there was a more protective effect exhibited by the vitamin D solution in the wildtype parental generation overall.

Using the mean values for the adult body mass assay and wing size/area assay, as well as the values for the glucose and sugars content assay, we conducted t-tests to show statistically significant results. These values were compared to the values of that seen in the fly groups reared on normal diets (control) and HSDs. For example, we compared wildtype parental generation flies reared on normal diets vs. wildtype parental generation flies reared on HSDs (WTPND vs. WTPHSD), wildtype parental generation flies reared on HSDs vs. wildtype parental generation flies reared on HSDs infused with 0.035 µL/mL 25(OH)D (WTPHSD vs. WTPHSD w/ 0.035), wildtype parental generation flies reared on HSDs vs. wildtype parental generation flies reared on HSDs infused with 0.045 µL/mL 25(OH)D (WTPHSD vs. WTPHSD w/ 0.045), and wildtype parental generation flies reared on HSDs vs. wildtype parental generation flies reared on HSDs infused with 0.055 µL/mL 25(OH)D (WTPHSD vs. WTPHSD w/ 0.055).

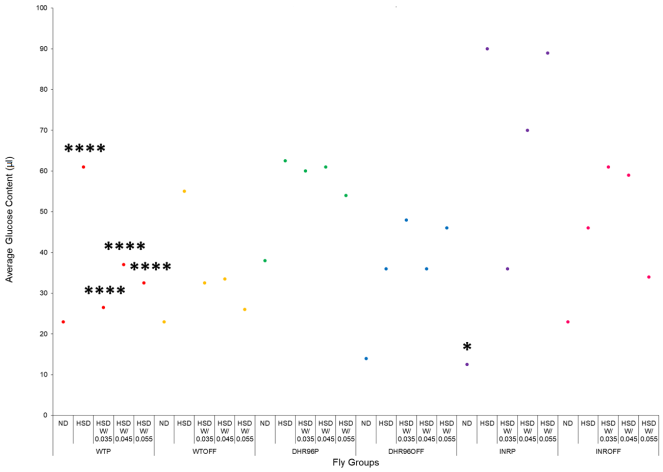

Figure 3: Wildtype parent flies fed HSDs had greater glucose content than did fly groups supplemented with 25(OH)D. A rapid colorimetric method with anthrone reagent was utilized to determine glucose content in an average of ten fruit flies. Statistically significant t-test results were obtained for the wildtype parental generation flies comparing HSD (WTPHSD) to that with varying concentrations of the vitamin D solution (WTPHSD W/ 0.035, WTPHSD W/ 0.045, WTPHSD W/ 0.055) (0.035 µL/mL, ****$p < 0.0001$; 0.045 µL/mL, ****$p < 0.0001$; 0.055 µL/mL, ****$p < 0.0001$).

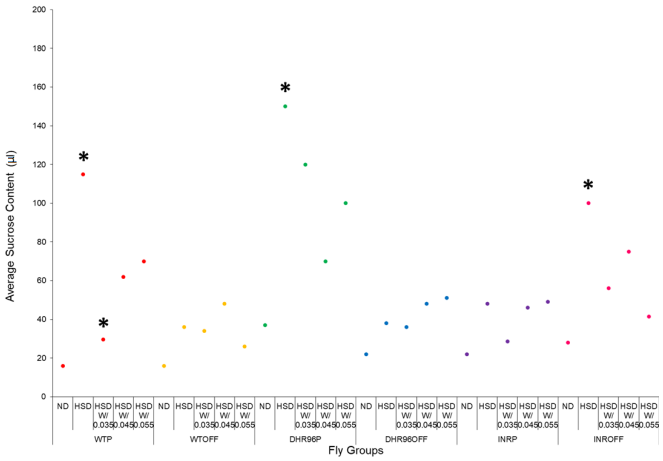

Figure 4: Wildtype parent flies fed HSDs had greater sucrose content than did fly groups supplemented with 25(OH)D. A rapid colorimetric method with anthrone reagent was utilized to determine sucrose content in an average of ten fruit flies. Statistically significant t-test results were obtained for the wildtype parental generation flies comparing HSD (WTPHSD) to that with the lowest concentration of the vitamin D solution (WTPHSD W/ 0.035) (0.035 µL/mL, *$p < 0.05$).

Therefore, the hypothesis was not fully supported because the highest concentration of the vitamin D solution was not the most effective in all instances or did not yield statistically significant improvements. However, overall we concluded that all three concentrations of the vitamin D solution administered to the flies reared on HSDs had a protective effect to varying extents. The tests for statistical significance show that the vitamin D had a more protective

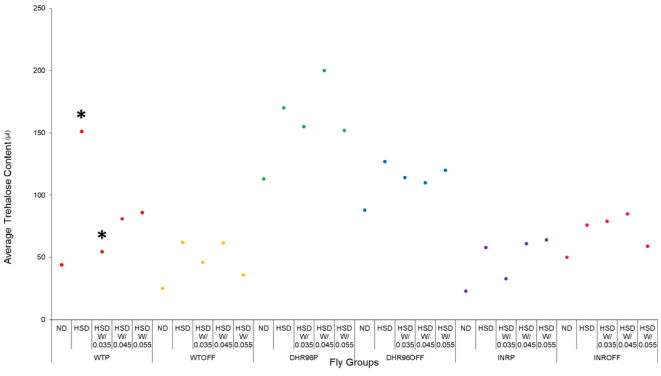

Figure 5: Wildtype parent flies fed HSDs had greater trehalose content than did fly groups supplemented with 25(OH)D. A rapid colorimetric method with anthrone reagent was utilized to determine trehalose content in an average of ten fruit flies. Statistically significant t-test results were obtained for the wildtype parental generation flies comparing HSD (WTPHSD) to that with the lowest concentration of the vitamin D solution (WTPHSD W/ 0.035) (0.035 µL/mL, *p < 0.05).

effect across the wildtype parental generation than with any other fly group tested (DHR96 mutant or InR mutant) and throughout the assays performed.

DISCUSSION

The results of this study did not support the proposed alternative hypothesis; statistical analyses performed led to the null hypothesis being rejected in some instances and accepted in others based on the results of the assays performed. We hypothesized that the highest concentration of the 25(OH)D solution (0.055 µL/mL) would be most effective in preventing the appearance of diabetic-like phenotypes (reduced adult body mass, wing size, elevated glucose, sucrose, and trehalose content in the flies). Results showed that all three concentrations of the vitamin D solution were effective in doing so, to varying degrees; the highest concentration of the vitamin D solution administered was somewhat effective in accomplishing this, even though there were only slight improvements in certain instances and when compared to the flies reared only on HSDs. Furthermore, the DHR96 mutant flies — those that lack a vitamin D receptor and served as the negative control — had reduced body masses (regardless of the vitamin D solution infused in the HSDs), reduced average wing size, and elevated sugar levels (7). In most group comparisons, the DHR96 mutant fly results were not statistically significant (as expected). Literature suggests that the flies reared on HSDs will have reduced body masses and wing sizes. We consistently observed this throughout the experiments conducted in this study. For the average adult body mass assay, the body masses of the offspring of the wildtype flies seemed to be reduced in comparison to those reared on normal diets; the vitamin D supplementation helped in keeping the reduced body mass from worsening to the extent of the reduced body masses observed in flies fed only HSDs (used to induce diabetic-like phenotypes). The results of the wing size assay indicated that the wildtype and InR mutant flies' offspring had damaged and/or decreased wing sizes (overall) when reared only on HSDs. The offspring given the vitamin D solution infused in the HSD, had wing sizes that were increased in comparison to those reared on HSDs; although a marginal effect overall, the vitamin D solution played a more protective role in the wildtype parental generation. This increase in wing size may be due to the regenerative effects that the vitamin D solution is involved in with muscle regeneration after injury and increasing cellular turnover (12). The mechanism by which the vitamin D solution exhibits restorative effects should be looked into. Glucose and sucrose concentrations were significantly different between the wildtype and InR mutant parent generations of flies reared on HSDs and high-sucrose with the varying concentrations of the vitamin D solution administered. Trehalose concentration was statistically significant only in the wildtype parental generation. Overall, all three concentrations of the vitamin D solution helped to restore the normal phenotypes or improve them from the diabetic-like phenotypes observed to different extents in the parental and offspring generations, but t-tests conducted showed that a statistically significant difference was observed most frequently in the wildtype parental generation.

It may be interesting to expand this epigenetics study with a heavy focus on the offspring, to incorporate a focus on data that was collected for the parental generation as well, given that the vitamin D solution was playing a protective role early on with parent flies across the fly groups. Lower concentrations of the vitamin D solution may be more effective in the parents, but higher concentrations of the vitamin D solution may be needed to observe a significant effect in the offspring. The research conducted in this study has applications in that further experimentation to target specific genes associated with T2D and DNA methyltransferase (DNMT) activity can be investigated to individualize treatments with the vitamin D solution. DNA methyltransferases are a group of enzymes that inhibit gene transcription and DNA methylation serves as an important epigenetic mark to assess. Future studies can try to use a wider range of concentrations of the vitamin D solution to determine the optimal concentration at which the solution is most effective in protecting against T2D. It should be noted that vitamin D overdose may lead to vitamin D toxicity and can be diagnosed with markedly elevated 25(OH)D concentrations (>150 ng/mL) accompanied by severe hypercalcemia and hypercalciuria and by very low or undetectable parathyroid hormone activity, in humans (13). Additionally, experimentation comparing the effects of vitamin D supplements to that of this specific metabolite of vitamin D, 25-hydroxyvitamin D3, on protecting epigenetic modifications associated with T2D can help determine which of the two is a more effective solution. Research comparing the effects of 25-hydroxyvitamin D3 to that of current drugs being used to treat the symptoms associated with T2D, such as metformin, can also be studied to understand which is a more

beneficial treatment. Additional experimentation is required for reproducible results. Further research needs to be done to gather conclusive evidence in support of 25-Hydroxyvitamin D3's protective role in the epigenome and its potential as a T2D treatment.

MAERIALS & METHODS

To rear flies on corresponding diets, stock solutions for the 25(OH)D solution (Sigma Aldrich 739650) were prepared. We raised the wildtype fly groups (Carolina Biological Supply Company) and DHR96 mutant fly groups (Bloomington Drosophila Stock Center) on corresponding diet vials, with 4.7 g of sucrose added to those on HSDs (Figure 6). Within each of these fly groups (wildtype, DHR96 mutants, and InR mutants), there were subgroups that consisted of flies reared on a normal diet, high-sucrose diet, and HSDs with the vitamin D solution infused (concentrations of 0.035 µL/mL, 0.045 µL/mL, and 0.055 µL/mL). Both parents and F1 offspring (of the wildtype, DHR96 mutants, and InR mutants) were reared on HSDs with the vitamin D solution infused (experimental conditions). These concentrations were determined based on the results of a study performed in which it was concluded that higher plasma 25(OH)D concentrations (> 30 ng/mL) were associated with lower hazard ratios for diabetes and even lower hazard ratios associated with 25(OH)D concentrations > 50 ng/mL (4). We stored the 25(OH)D solution at -20 ° Celsius, as soon as it arrived. We prepared the working concentrations of the solution by diluting in water. We administered the vitamin D solution by using it to hydrate the fly medium; it was mixed well to ensure all parts of the medium were penetrated.

The next step involved performing the cross to obtain viable InR mutants (Figure 6). They served as T2D models that differ in that these flies were born with some form of the mutations causing diabetic-like phenotypes in comparison to the wildtype model that was reared on HSDs to induce diabetic-like phenotypes. Within 8–10 hours of eclosion, we collected virgin females from one stock: In(3R)GC25, InR[93Dj-4]/TM3, Sb[1] (Bloomington Drosophila Stock Center) and crossed with males from another stock: InR[E19]/TM2 (Bloomington Drosophila Stock Center). To collect virgin females, we anesthetized and sexed them using FlyNap (Carolina Biological 173010). 25% of the progeny should be In(3R)GC25, InR[93Dj-4]/InR[E19]. In(3R)GC25, InR[93Dj-4]/InR[E19] are the viable mutants. Viability is classified as the ability to survive until adulthood; it was crucial to obtain viable mutants to be able to perform assays after experimentation. The viable mutants had wildtype body color and normal bristles. InR[E19]/TM3, Sb[1] had short stubbly bristles (easy to see under a microscope). In(3R)GC25, InR[93Dj-4]/TM2 had a partial transformation of halteres (the balancing organ of the fly, seen as either of a pair of knobbed filaments that take the place of the hind wings, vibrating during flight) to wings. TM2/TM3, Sb[1], if viable, had short stubbly bristles, the haltere phenotype, and a dark body color. Progeny with short, stubby bristles and haltere phenotype were discarded and those with wildtype body color and normal bristles were kept, as they were viable mutants. These flies grew under normal conditions and diet at 25° Celsius before being reared under the experimental conditions (HSD, vitamin D solution) to ensure that the temperature and time-sensitive diabetic-like phenotypes were observed. InR mutant fly groups were then reared on corresponding diet vials, keeping in mind that adult body mass and wing size assays were to be performed between Days 9 and 13.

The first assay conducted involved measuring the adult body mass (Figure 6). We measured body mass in euthanized individual flies by placing on a scale (average). Pictures were taken.

Another assay conducted involved measuring the size/area of the wings (Figure 6). We dissected the fly wings, placed them carefully on grid paper, took pictures, and analyzed using ImageJ software.

The glucose and sugars content assays were based on an established protocol used in this study (14) (Figure 6). We prepared standard solutions for glucose, sucrose, and trehalose (1 mg/mL) in 25% as well as anthrone reagent (used to measure carbohydrates). To make a calibration curve, 25, 50, 100, 150, and 200 ug glucose, sucrose, and trehalose solution (1 mg/mL) were placed in tubes, filed with anthrone reagent to the mark, mixed, and heated in the tube heater (Southwest Science). After letting it cool, the OD was read at 625 nm. Place a fruit fly in a culture tube, add 0.2 ml sodium sulfate solution, and crush ten fruit flies with a glass rod. Add 0.5 ml methanol, mix (vortex) well and centrifuge for about 1 minute. Decant the supernatant (containing the sugars) to

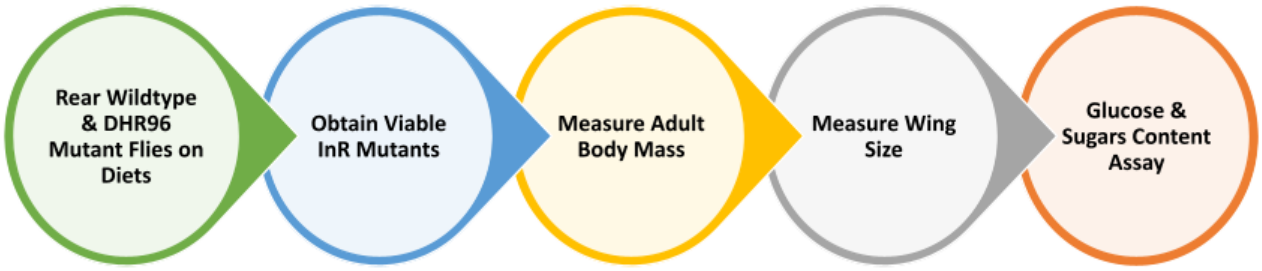

Figure 6: Simplified Procedures Schematic.

a second set of tubes and evaporate the solvent in the tube heater down to 0.1-0.2 ml. Glucose remains behind in the first tube, adsorbed on the precipitated sodium sulfate, along with fly tissue. Fill both tubes to the mark with the anthrone reagent mix, heat for 17 minutes, cool, mix and determine OD at 625 nm. Dilute the high ODs if needed. Glucose content per ten flies can be read directly from the calibration line. Repeat these steps to determine sucrose and trehalose content per ten flies.

REFERENCES

1. "Type 2 Diabetes." Centers for Disease Control and Prevention, Centers for Disease Control and Prevention, 30 May 2019.
2. Gallagher, J. Christopher, and Daniel D. Bikle. "Vitamin D: Mechanisms of Action and Clinical Applications." Endocrinology and Metabolism Clinics of North America, vol. 46, no. 4, 2017, pp. xvii-xviii., doi:10.1016/j.ecl.2017.09.001.
3. Park, Sue K., et al. "Plasma 25-Hydroxyvitamin D Concentration and Risk of Type 2 Diabetes and Pre-Diabetes: 12-Year Cohort Study." PLOS ONE, vol. 13, no. 4, 2018, doi:10.1371/journal.pone.0193070.
4. Wolden-Kirk, Heidi, et al. "Vitamin D and Diabetes: Its Importance for Beta Cell and Immune Function." Molecular and Cellular Endocrinology, vol. 347, no. 1-2, 2011, pp. 106–120., doi:10.1016/j.mce.2011.08.016.
5. Norman, A., et al. "Vitamin D Deficiency Inhibits Pancreatic Secretion of Insulin." Science, vol. 209, no. 4458, 1980, pp. 823–825., doi:10.1126/science.6250216.
6. Graham, P., and L. Pick. "Drosophila as a Model for Diabetes and Diseases of Insulin Resistance." Current Topics in Developmental Biology, 2017, pp. 397–419., doi:10.1016/bs.ctdb.2016.07.011.
7. Murillo-Maldonado, J. M., et al. "Drosophila Insulin Pathway Mutants Affect Visual Physiology and Brain Function Besides Growth, Lipid, and Carbohydrate Metabolism." Diabetes, vol. 60, no. 5, 2011, pp. 1632–1636., doi:10.2337/db10-1288.
8. Ling, Charlotte, and Tina Rönn. "Epigenetics in Human Obesity and Type 2 Diabetes." Cell Metabolism, vol. 29, no. 5, 2019, pp. 1028–1044., doi:10.1016/j.cmet.2019.03.009.
9. Davegårdh, Cajsa, et al. "DNA Methylation in the Pathogenesis of Type 2 Diabetes in Humans." Molecular Metabolism, vol. 14, 2018, pp. 12–25., doi:10.1016/j.molmet.2018.01.022.
10. Rönn, Tina, and Charlotte Ling. "DNA Methylation as a Diagnostic and Therapeutic Target in the Battle against Type 2 Diabetes." Epigenomics, vol. 7, no. 3, 2015, pp. 451–460., doi:10.2217/epi.15.7.
11. Shingleton, Alexander W, et al. "The Temporal Requirements for Insulin Signaling During Development in Drosophila." PLoS Biology, vol. 3, no. 9, 2005, doi:10.1371/journal.pbio.0030289.
12. Owens, Daniel J., et al. "A Systems-Based Investigation into Vitamin D and Skeletal Muscle Repair, Regeneration, and Hypertrophy." American Journal of Physiology-Endocrinology and Metabolism, vol. 309, no. 12, 2015, doi:10.1152/ajpendo.00375.2015.
13. Marcinowska-Suchowierska, Ewa, et al. "Vitamin D Toxicity: A Clinical Perspective." Prime Archives in Endocrinology, 2020, doi:10.37247/paendo.1.2020.8.
14. Handel, E Van. "Rapid Determination of Glycogen and Sugars in Mosquitoes." Journal of the American Mosquito Control Association, vol. 1, no. 3, 1985, pp. 299–301.

Article submitted: March 12, 2019
Article accepted: May 3, 2019
Article published: September 20, 2021