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Development of Diet-Induced Insulin Resistance in *Drosophila melanogaster* and Characterization of the Anti-Diabetic Effects of Resveratrol and Pterostilbene

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Summary

As the prevalence of type II diabetes mellitus (T2DM) has increased, so has the need to further examine the disorder's underlying features and potential treatment options. The common fruit fly *Drosophila melanogaster* has proven to be a convenient model organism to study T2DM, but whether it can serve as a model organism to identify T2DM treatment remains unanswered. To answer this question, we attempted to establish a T2DM model in *Drosophila*. We induced insulin resistance (IR) in *Drosophila* through high sucrose diet (HSD) and characterized the therapeutic effects of the polyphenols Resveratrol and Pterostilbene. *Drosophila* pupation rates were evaluated for signs of developmental delay. Physical activity tests that measured 3rd instar larvae crawling rates were performed. Upon the verification of HSD effects on *Drosophila* phenotype, assays incorporating the polyphenolic treatment groups were performed. Statistically significant results from the study include a developmental delay, decreased physical activity in HSD larvae, and increased weight and glucose concentration levels in HSD-fed adult *Drosophila*. Resveratrol and Pterostilbene treatment notably overturned the weight gain and glucose levels, while preliminary results from the real-time PCR and oxidative stress resistance assays were inconclusive. Altogether, the results of this study suggest that *Drosophila* can be utilized as a model organism to study T2DM and novel T2DM pharmacological treatments.

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Introduction

The metabolic disorder type II diabetes mellitus (T2DM) has become increasingly prevalent in recent years, with the latest statistics indicating that nearly 30.3 million people are affected in the US alone (1). T2DM is characterized by a decrease in cellular sensitivity to the insulin peptide hormone, resulting in a detrimental accumulation of glucose in the bloodstream where it is unable to be utilized for metabolism and storage purposes. High blood glucose levels have the potential to trigger fatal cardiovascular diseases, extensive organ damage, and neuropathy (2). Given the rising rates of T2DM and the severe health implications associated with it, the need to characterize and apply potent therapeutic agents has increased tremendously.

The fruit fly *Drosophila melanogaster* can serve as an effective model organism for the pathology of T2DM and as a model organism to analyze the therapeutic effects of polyphenols such as Resveratrol and Pterostilbene. *Drosophila* is a cost-effective and convenient model organism amenable to screening through libraries of chemical compounds for therapeutic purposes in consistent and reproducible assays. Although *Drosophila* and humans do not share key organs for insulin production and metabolism, both organisms do contain several organs that are homologous. Organs in the *Drosophila* model that correspond to those in humans include the Malpighian tubules and nephrocytes (kidney), the dorsal vessel (heart), the fat body (liver and adipose), and the hemolymph (blood and gastrointestinal tract) (3). In addition, the *Drosophila* neuroendocrine system shares many major similarities with its mammalian equivalent. Insulin producing cells (IPCs) that resemble pancreatic β-cells can be found within the *Drosophila* brain. Although insulin is not present in *Drosophila*, these cells secrete seven functionally homologous insulin-like peptides (dILP1, dILP2, dILP3, dILP4, dILP5, dILP6, dILP7) into the hemolymph (4,5). Likewise, cells in the *Drosophila* model resembling pancreatic α cells that counterbalance insulin can be found near the dorsal vessel (4).

At the molecular level, *Drosophila melanogaster* is an effective model organism for T2DM due to its key similarities with mammals in terms of the insulin/insulin-like growth factor signaling (IIS) pathway (**Figure 1**). This

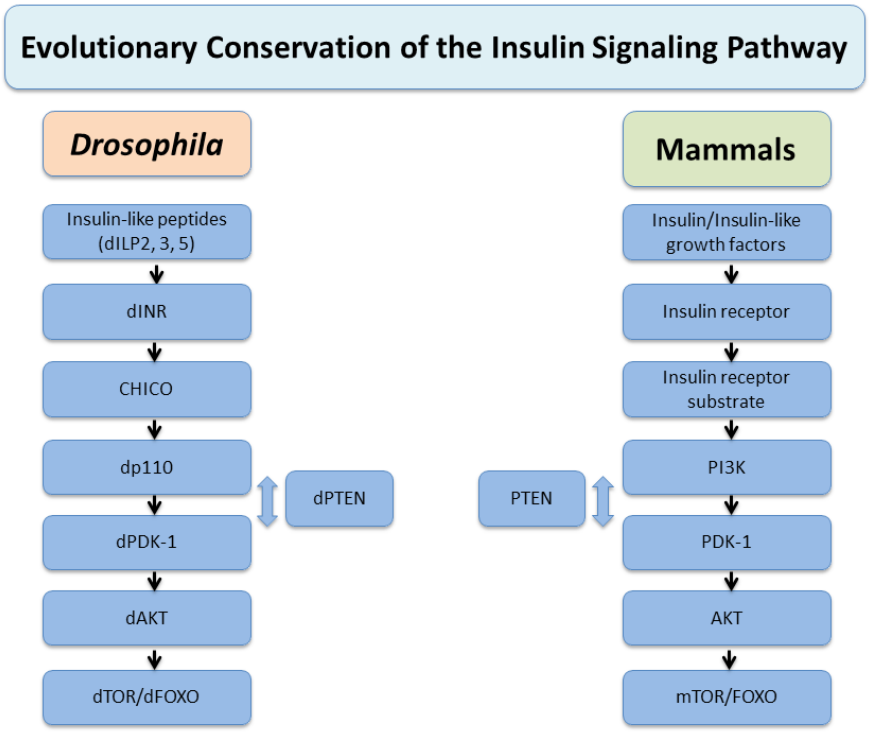

Figure 1. Diagram of the evolutionary conservation of the insulin signaling pathway in *Drosophila* and mammals.

intracellular cascade is regulated by specialized protein kinases that transduce signals through phosphorylation (4). While there are separate pathways for insulin and the insulin-like growth factor in humans, the *Drosophila* IIS pathway is a combined network that mediates both metabolism and growth (4). dILP (*Drosophila* insulin-like peptide) binds to the *Drosophila* insulin-like receptor protein (dINR) within the cell membrane, triggering the pathway's initiation (4). dINR subsequently activates the IIS pathway by phosphorylating the growth-controlling protein CHICO, a homolog of the insulin-receptor substrate (IRS) in mammals (6). Conserved targets that are phosphorylated downstream of CHICO include Dp110 (PI3K in mammals) and AKT. AKT can phosphorylate both dFOXO, a transcriptional effector of insulin signaling and lipid metabolism, and dTOR, a metabolic enzyme involved in sugar uptake (4). Defects such as the compromised expression of major insulin-signaling proteins, including dINR, can induce the common phenotypes of T2DM. In addition to the conserved positive IIS pathway regulators in *Drosophila*, many conserved negative regulators are also present. dPTEN acts antagonistically towards Dp110, just as PTEN and PI3K interact in the mammalian insulin-signaling pathway (7). The main negative regulator of the IIS pathway is a lipocalin called Neural Lazarillo (NLaz). NLaz is the functional equivalent of the mammalian Retinol Binding Protein (RBP4) and a target of the conserved c-jun N-terminal kinase (JNK) pathway (8). While the moderate expression of *NLaz* is key to maintaining metabolic homeostasis in *Drosophila*, its overexpression has been found to initiate *dILP* resistance and T2DM (8).

The polyphenols Resveratrol and Pterostilbene have emerged as promising therapeutic agents for T2DM. These anti-oxidants, produced by various plants upon microbial infections, can be found in red wine, peanuts, and blueberries (9). While Pterostilbene shares similar anti-diabetic effects with Resveratrol, the two polyphenols differ in terms of bioavailability and *in vivo* potency (10). Due to its two methoxy groups, Pterostilbene is more lipophilic and thus better ingested orally (11). After absorption, the two polyphenols neutralize free radicals that can trigger destructive oxidative stress (12). Both Resveratrol and Pterostilbene function as calorie-restriction mimetics, mediating physiological effects that mimic a low-calorie diet (9). While the two polyphenols are classified as dietary supplements, experimental studies have indicated that they may also have anti-cancer, anti-neurodegenerative, and anti-inflammatory effects (9,11). Resveratrol activates the *SIRT1* gene (*dSir2* in *Drosophila*) that is linked to increased metabolism and insulin signaling (9). In *Drosophila*, *dSir2* functionality has been found

to be analogous to its mammalian counterpart, as a knockdown of the gene leads to increased hemolymph *dILP* expression and decreased IIS signaling (13). Aside from its ability to increase *SIRT1* expression, Resveratrol also activates the AMP-activated protein kinase (AMPK) enzyme (14). AMPK, a key modulator of cellular energy homeostasis, works by reducing the activity of ATP consuming pathways and increasing the activity of ATP producing pathways (14). Pterostilbene possesses potential anti-diabetic effects similar to Resveratrol, but further experimentation is required to determine whether the two polyphenols could serve as effective means of anti-diabetic treatment. A model organism-based characterization and comparison of the polyphenols' anti-diabetic effects could assist in the search for novel therapeutic approaches to T2DM.

Results

Developmental time

To study the effects of the high sucrose diet (HSD) on *Drosophila* larval development, *Drosophila* larvae (n=45 per group), were randomly placed into groups containing identical proportions of larvae at different instar stages. Each group was placed into a vial containing either the control medium or the HSD medium. Larval pupariations were recorded in each vial for 10 days, and pupariations rates were calculated based on the percentage of the initial 45 larvae that had pupariated. Notably, *Drosophila* larvae reared on the HSD demonstrated significantly delayed development (**Figure 2A-B**).

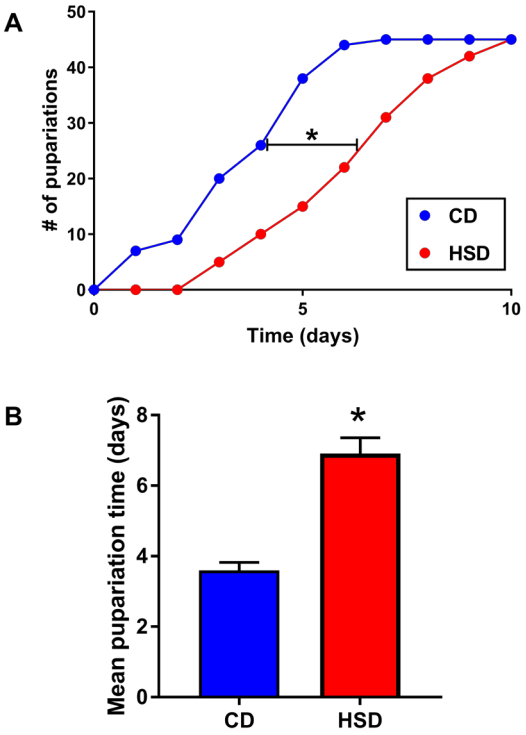

Figure 2. Effect of the HSD on *Drosophila* larval developmental time. 2nd-3rd instar larvae were obtained from HSD and CD groups (n=45 per group) and were transferred to fresh vials containing either the HSD or the CD. Vials were observed daily for up to 10 days in order to record pupariations. (A) Curves were compared with the extra sum-of-squares F test [*F (DFn, DFd)* = 24.38 (1,14)], *: *p*=0.0002. (B) Mean pupariation time was calculated from EC50 values and expressed as mean ± SE, *p*=0.0002.

Physical activity

To determine the effects of the HSD on physical endurance within the *Drosophila*, we recorded larval physical activity rates. Third instar larvae reared on the control diet (CD) and the HSD were individually transferred to petri dishes containing a dilute yeast paste solution. After a 30-second adjustment period to allow the larvae to adjust to the new surroundings, the number of larval peristalsis (whole-body contractions) was recorded for thirty seconds underneath a stereomicroscope. *Drosophila* larvae reared on the HSD had significantly lower rates of peristalsis contractions (**Figure 3**).

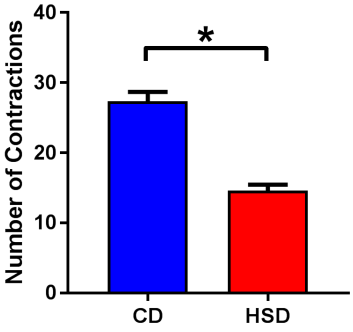

Figure 3. Effects of the HSD on *Drosophila* larval physical activity. 3rd instar larvae from both dietary groups (n=30 per group) were obtained, rinsed, and placed on yeast-paste containing petri dishes underneath a stereomicroscope. Peristalsis contractions were counted for 30 seconds for each individual larva. Data were analyzed by unpaired t-test (*p*=0.0013).

Circulating glucose concentration

To investigate the potentially deleterious effects of the HSD on circulating glucose concentration levels as well as the potentially protective effects of Resveratrol and Pterostilbene treatment, we measured glucose concentration levels within the different groups. HSD-fed *Drosophila* adults had significantly higher circulating glucose concentration levels than CD-fed *Drosophila* adults (**Figure 4**). *Drosophila* adults that underwent the

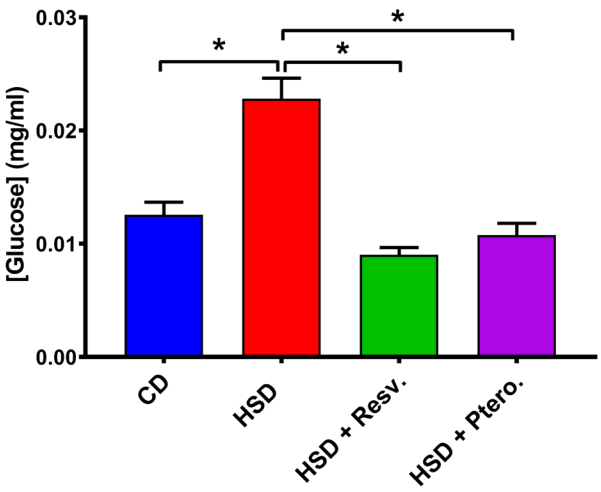

Figure 4. Effects of the HSD and the antioxidants Resveratrol and Pterostilbene on *Drosophila* circulating glucose concentration. Circulating glucose concentration was assayed in twelve samples per group. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc tests; *: $p \leq 0.0001$.

6-day treatment with either Resveratrol or Pterostilbene had significantly lower circulating glucose concentration levels than controls, indicating that Resveratrol or Pterostilbene treatment may have restorative and protective effects with respect to insulin signaling (**Figure 4**).

Weight

To determine the effects of the experimental diets on *Drosophila* weights, we obtained groups of *Drosophila* adults were and measured their collective weights on an analytical balance. Each group consisted of 10 adults (5 male, 5 female), with each sex being weighed separately. We found that HSD-fed *Drosophila* male and female adults had higher-than-average weights than the male and female *Drosophila* adults fed the CD (Fig. 5). Notably, male and female *Drosophila* administered either Resveratrol or Pterostilbene for a 6-day period were found to weigh less than *Drosophila* reared on the HSD (**Figure 5**). This difference was significant for HSD-fed males and Resveratrol-treated males, HSD-fed females and Resveratrol-treated females, and HSD-fed females and Pterostilbene treated females (**Figure 5**).

Gene Expression

To determine the utility of *Drosophila* with respect to modeling insulin resistance and anti-diabetic modes of treatment at the molecular level, we analyzed gene expression of key components of the insulin-signaling pathway. Total RNA was extracted from groups of at least 30 adult females and gene expression was measured by qRT-PCR. The expression of seven genes, all of which are involved in either insulin signaling, JNK-signaling, or systemic inflammation, were analyzed (**Figure 6A-G**). Notably, HSD-fed *Drosophila* adults had a significantly lower expression level of *dSir2* relative to *Drosophila* adults in the other three dietary groups (**Figure 6A**). Although HSD-fed and Resveratrol-treated adults had increased *FASN1* expression, the gene was slightly downregulated in Pterostilbene-treated adults (**Figure 6D**). However, the observed result was not statistically significant (5), and higher sample sizes or altered treatment timeline may be needed to determine the real effect of Pterostilbene treatment.

Oxidative stress resistance

In order to determine the effects of the HSD on oxidative stress resistance and to evaluate the antioxidant capacities of Resveratrol and Pterostilbene, we carried out survivorship assays involving conditions of oxidative stress. Hydrogen peroxide (1.5%) was used to trigger oxidative stress. Post-eclosure, *Drosophila* adults were aged for 6 days in vials containing each of the experimental diets before being transferred to new vials containing hydrogen peroxide in an agar matrix. The vials were observed twice daily in order to determine the number of deceased *Drosophila*. *Drosophila* in both polyphenolic treatment groups had higher rates of survivorship than those on the CD, suggesting some degree of protection or biological compensation to the pre-existing stress **(Figure 7**).

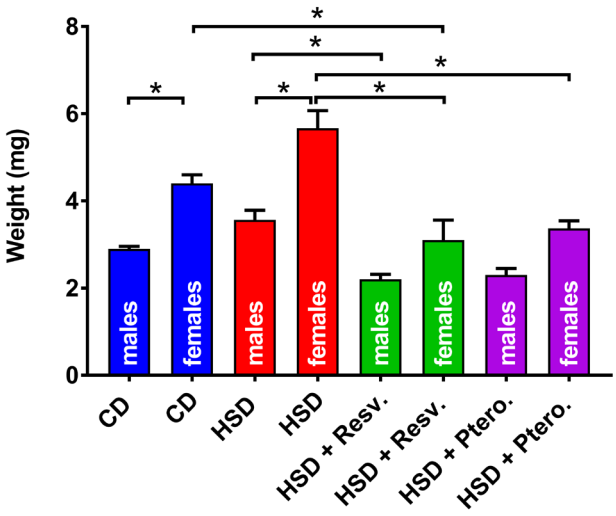

Figure 5. Effects of the HSD and the antioxidants Resveratrol and Pterostilbene on *Drosophila* weight. *Drosophila* adult flies of each gender (n=5) were weighed. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc tests; *: $p<0.05$.

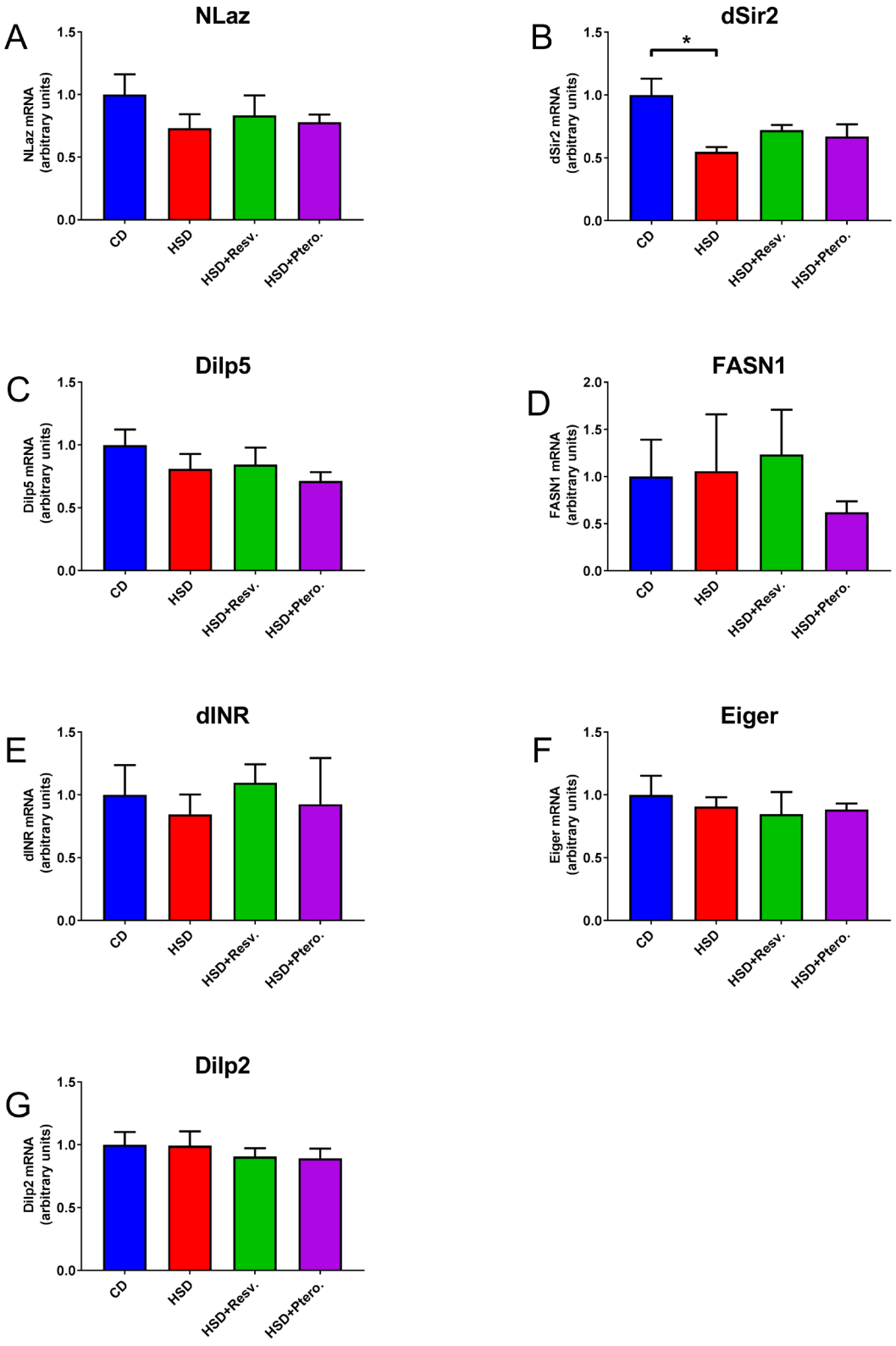

Figure 6. Effects of the HSD and the antioxidants Resveratrol and Pterostilbene on the expression of *Drosophila* genes involved in insulin signaling, JNK-signaling, and systemic inflammation. Total RNA was extracted from sets adult female *Drosophila* (n=4-6), gene expression was quantified by Real-time PCR and standardized relative to the housekeeping gene *RPL32*. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc tests; *: $p<0.05$.

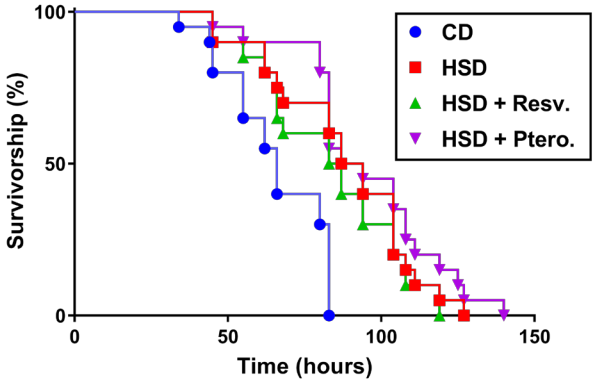

Figure 7. . Effects of the HSD and the antioxidants Resveratrol and Pterostilbene on oxidative stress resistance in *Drosophila*. Adult *Drosophila* (5 females, 5 males) from each of the four dietary groups, after being aged for 6 days in vials containing each of the four experimental diets, were transferred to fresh vials containing 1.5% H2O2 in a 2.0% agar matrix. The vials were observed twice daily to determine the number of deceased adult *Drosophila*. Data were analyzed with the Log-rank (Mantel-Cox) test. CD vs. HSD, p=0.0002; CD vs. HSD + Resv, p=0.0017; CD vs. HSD + Ptero, p<0.0001.

Discussion

Induction and treatment of T2DM and IR at the molecular level

The results of this study's genetic analysis component demonstrate the potential utility of *Drosophila* as a model organism for insulin resistance and T2DM. Notably, flies raised on the HSD had a lower mean expression of *dSir2* relative to flies in the CD group (p=0.0489); additionally, this *dSir2* HSD expression level was lower than that of flies in the Resveratrol and Pterostilbene treatment groups. *dSir2* is a functional homolog of the *SIRT1* gene in humans that is linked to cellular metabolism. In the *Drosophila* model, *SIRT1* knockdown has been proven to lower the activity of the insulin (dILP)-signaling pathway and extend lifespan (8,13,17). The *dSir2* deficiency found in this study indicates that cellular metabolism and insulin-signaling may have been compromised in the flies due to the 1.0M HSD; furthermore, the higher expression levels of *dSir2* in the treatment groups suggest that Resveratrol and Pterostilbene treatment may have had an offsetting effect on *dSir2* downregulation.

Aside from *dSir2* expression, the HSD did not appear to have a pronounced effect on specific insulin-signaling pathway genes. The expression of *dINR*, the *Drosophila* homolog of the mammalian insulin receptor gene, was slightly downregulated in HSD flies at a comparatively higher rate than that of flies in the CD and polyphenolic treatment groups. However, due to the lack of statistical significance, further experimentation is needed to clarify the effects of the HSD and polyphenolic treatment on *dINR* expression. HSD consumption did appear to slightly lower the expression of *dILP5*, a Drosophila homolog of mammalian insulin, although this effect was not noticeably countered by Resveratrol or Pterostilbene treatment.

The HSD slightly increased the mean expression level of *FASN1* (fatty acid synthase 1), a gene involved in fatty acid synthesis. In an insulin resistant state, higher levels of free fatty acids can leave cells within the adipose tissue and be taken up by organs that are unable to safely store excessive amounts of fat (9). Insulin functions as an anti-lipolytic hormone in adipose tissue that can decrease the release of free fatty acids. Resistance to insulin by fat cells leads to dangerously high levels of circulating free fatty acids, as the release of free fatty acids are no longer repressed (9). Given the role of free fatty acids in mediating insulin resistance and systemic inflammation, the increased expression of the fatty acid synthase in HSD flies is notable (9). Only Pterostilbene appeared to substantially reduce the expression of this gene. Although significant changes in expression were not observed for inflammation-mediating genes *NLaz* (*RBP4*) and *Eiger* (*TNF-α*) or for the insulin-signaling gene *dILP2*, notable results involving the remaining genes indicate that *Drosophila* may be a viable model for insulin resistance at the molecular level. Further studies involving significantly higher sample sizes may need to be carried out in order to determine the specific roles of *dINR*, *NLaz*, *Eiger*, and *dILP2* with respect to inflammation and insulin-signaling in the Drosophila model.

Induction and treatment of T2DM and IR at a broad, phenotypic level

Our results validate the use of *Drosophila* as a model organism for the induction and treatment of T2DM. As observed in the developmental time assay, *Drosophila* larvae that were raised on the 1.0M HSD exhibited significantly lower rates of growth and development relative to larvae raised on the CD. Notably, as the *Drosophila* insulin-signaling pathway has been found to modulate growth in addition to metabolism, any growth disruptions in *Drosophila* can be linked to defects in the insulin-signaling pathway (4).

Another important phenotype associated with T2DM/IR and obesity in mammals is compromised physical endurance. This phenotype results from an accumulation of circulating glucose that inhibits the major energy molecule from being properly utilized by cells. Results from this study prove that *Drosophila* larvae fed the 1.0M HSD had significantly lower rates of physical activity than CD-fed larvae, suggesting a

systemic failure in being able to properly metabolize digested nutrients. *Drosophila* adults raised on the 1.0M HSD weighed more compared to their counterparts in the control group. Elevated circulating glucose levels provide a strong indication that the HSD triggered insulin resistance within the *Drosophila*, especially given that increased extracellular glucose levels are a hallmark phenotype associated within insulin resistance and diabetes mellitus. Resveratrol and Pterostilbene helped to curb both weight gain and increased glucose levels. These effects may be attributed to the polyphenols' ability to offset the downregulation of genes involved in metabolism and insulin (dILP)-signaling, such as *dINR* and *dSir2* (**Figure 6**).

Given the role of insulin resistance in increasing adiposity and free fatty acid circulation in mammals, the weight gain in HSD-fed *Drosophila* is a notable result in terms of validating the utility of *Drosophila* as a model for diabetes mellitus (15). The comparatively lower weight levels observed in the Resveratrol and Pterostilbene treated *Drosophila* suggest that the two polyphenols may have protective effects with respect to weight and insulin signaling, and that their protective effects can be modeled in *Drosophila*.

We initially hypothesized that the HSD-fed flies would have the lowest survivorship in H2O2 stress conditions, while Resveratrol and Pterostilbene treatment would increase the survivorship of flies placed in similar conditions. This assumption was based on the role of ROS (reactive oxygen species) in the development and pathophysiology of insulin resistance in humans. Because Pterostilbene and Resveratrol are antioxidants, we hypothesized that both could counteract ROS in the *Drosophila* model, thus increasing survivorship in oxidative stress conditions (16). Flies in both polyphenolic treatment groups had higher survival rates than those in the CD group, validating our initial hypothesis. Surprisingly, the CD-fed flies in both oxidative stress trials were found to have the least resistance to oxidative stress, displaying higher death rates than the flies in the HSD group. One possible explanation for this result could be that CD flies were not exposed to the HSD as larvae and freshly emerged adults, unlike flies in the other groups. Their physiological systems were not preconditioned with HSD and were less capable of handling the HSD-induced oxidative stress when exposed to it as mature adults. Additional experiments containing only CD-fed flies and flies fed varying concentrations of the HSD could be carried out to determine if there is a correlation between flies being fed the HSD as larvae and those flies having additional oxidative stress resistance as adults. Future experimentation with the parameters described in this study may necessitate that the HSD-fed *Drosophila* group serve as a control group for the flies treated with polyphenols. Larger sample sizes may be needed to obtain statistically significant results.

Implications for major diseases

The metabolic disorder T2DM is a major risk factor for long-term health complications such as cardiovascular disease, extensive organ damage, and poor nerve health (15). The increasing prevalence of the disorder and detrimental side effects associated with existing modes of treatment such as Metformin justify the need to find more safe and potent therapeutic options (18). Through this research, the polyphenols Resveratrol and Pterostilbene have been shown to counteract key negative physiological developments of T2DM, including increases in extracellular glucose concentration and body weight. At the molecular level, both polyphenols have been found to offset the downregulation of genes involved in insulin signaling (i.e. *dSir2* and *dINR*), although further research is needed to ascertain their effects on the other genes tested in this study. This study is the first to test therapeutic compounds in the diet-induced T2DM *Drosophila* model for the purpose of ameliorating the T2DM-induced deleterious phenotype. Polyphenols, such as the ones used in the current study or other ones, could potentially be used in the future as a primary treatment for diabetes mellitus, either replacing current forms of treatment or supplementing existing ones. *Drosophila melanogaster* has emerged as a cost-effective and convenient model in which anti-diabetic substances, including polyphenols, can be screened and tested prior to use in humans. Our results demonstrate that the *Drosophila* model could potentially supplant costlier and less convenient rodent models for T2DM and IR.

Methods

Drosophila stocks and culture

Stocks of the wild-type Oregon-R strain of *Drosophila melanogaster* were obtained from the Carolina Biological Supply Company. All flies were kept in a room with an ambient temperature of 20-22 °C. To produce the media, Formula 4-24 Instant *Drosophila* medium was mixed with either 12 mL of distilled water or 12 mL of 1.0 M sucrose solution; 5-6 grains of yeast were then added to the mediums.

For assays involving adult *Drosophila* in the 1.0 M HSD and CD groups, adult (non-virgin) flies were used; for the Resveratrol + 1.0 M HSD and Pterostilbene + 1.0 M HSD groups, adult flies, which were initially collected as virgins, were used for assays after undergoing treatment for a 6-day period.

Resveratrol and Pterostilbene solutions

Resveratrol and Pterostilbene solutions (1 mM)

were prepared. To prepare the Resveratrol solution, 3 mg of Resveratrol (EMD Millipore) was transferred to a beaker containing 13.14 mL of ethanol. The beaker was then placed upon a magnetic stirrer and the solution was agitated until the Resveratrol fully dissolved. For the Pterostilbene (EMD Millipore), 2 mg was transferred to a beaker containing 7.8 mL of ethanol. This beaker was placed upon a magnetic stirrer and its solution was agitated until the Pterostilbene fully dissolved. 200 µL of the final Resveratrol and Pterostilbene solutions were transferred to each individual treatment culture medium along with 12 mL of the 1.0 M HSD solution.

Real-time polymerase chain reaction (PCR)

At least 30 adult female *Drosophila* were obtained, transferred to a 50 mL plastic test tube, and homogenized in TRI reagent (MRC Gene). Total RNA was extracted from the solutions using the manufacturer's instructions. Extracted RNA was DNase treated with TURBO DNA-free kit (Invitrogen) and quantified using a Nanodrop Spectrophotometer (Thermo Scientific). The extracted RNA was then reverse transcribed into cDNA using the Superscript III Reverse Transcriptase kit (Invitrogen) per the manufacturer's instructions. Subsequently, 1 µL of the resulting cDNA was used for Real-time PCR analysis. Real-time PCR was conducted using the SYBR Green PCR master mix (Applied Biosystems), per the manufacturer's instructions, and measured on a CFX96 Real-Time PCR system (Bio-Rad). cDNA abundance was calculated by comparative CT relative to the housekeeping gene *RPL32*. Primers for *RPL32* and the target genes were obtained from FlyPrimerBank database and are reported in Table 1.

Gene (alias)	FlyBase Gene	FlyPrimerBank primer pair	Sequences (Forward, Reverse)	Amplicon size (bp)
Dilp2 (Ilp2)	*FBgn0036046*	PP36980	GGGTGTACTCAATTCCCTGGC CTTTTTGCAGCACCTCTCCAC	79
Dilp5 (Ilp5)	*FBgn0044048*	PP5930	CGCTCCGTGATCCCAGTTC AGGCAACCCTCAGCATGTC	112
dINR (InR)	*FBgn0013984*	PP12687	AAGCGTGGGAAAATTAAGATGGA GGCTGTCAACTGCTTCTACTG	148
dSir2 (Sirt1)	*FBgn0024291*	PD70442	CATTATGCCGCATTTCGCCA GAAGGTGTTCACTGAGGCCA	124
Eiger (egr)	*FBgn0033483*	PD70026	AGCTGATCCCCCTGGTTTTG GCCAGATCGTTAGTGCGAGA	72
FASN1	*FBgn0027571*	PP20769	TGACCAACAGTTCTTCGGTGT GCGTCAATAATAGCTTCATGGGT	99
NLaz	*FBgn0053126*	PP9095	TGCTGCTTATCTCCGTGGTAT CCAGTAGCTTAACATCTGGGC	90
RpL32	*FBgn0002626*	PD41811	GCCCAAGGGTATCGACAACA GCGCTTGTTCGATCCGTAAC	85

Table 1. Real-time PCR primers

Developmental time

Culture media was removed from the vials of two dietary groups (HSD 1.0 M and CD) and placed in labeled petri dishes containing distilled water. Fifteen third-instar larvae and thirty second-instar larvae were obtained from the petri dishes with plastic, disposable pipettes and placed into new vials containing either the 1.0M HSD or CD. Each day afterwards, the number of pupariations (larvae that form pupae) were recorded.

Physical activity

For 1.0M HSD and CD groups, 10 third-instar larvae were obtained and rinsed in 15 mL of distilled water. The rinsed larvae were then placed upon petri dishes to be viewed underneath a stereomicroscope at 40x; these petri dishes contained a diluted yeast paste solution (15.2 g yeast/100 mL water). A 20 second acclimatization period was observed before the data recording in order to allow the larvae to adjust to the yeast-paste solution. Following acclimatization, the number of whole-body peristalsis contractions were counted for each individual larva for 30 seconds.

Circulating glucose concentration assay

10 adult *Drosophila* (5 males, 5 females) were anesthetized and their sexes were determined. The flies were subsequently rinsed in distilled water and blot-dried dried with paper towels. Then, the flies were transferred to 1.5 mL Eppendorf tubes containing 0.5 mL deionized water and crushed with a pellet pestle. The resulting solution was centrifuged for 6 minutes at 3,300 rpm in a microcentrifuge. A sample blank was prepared by pipetting 10 µL of sample into a Eppendorf tube alongside 50 µL of deionized water. A reagent blank was prepared by pipetting 50 µL of Hexokinase reagent (Sigma-Aldrich) into an Eppendorf tube alongside 50 µL of deionized water. Finally, a test solution was prepared by pipetting 50 µL of Hexokinase reagent into a cuvette alongside 10 µL of sample. All solutions were mixed thoroughly and incubated at room temperature for 15 minutes. The absorbance of 2 µL of each sample was measured at 340 nm with a Nanodrop Spectrophotometer (Thermo Scientific) blanked with deionized water. Calculations were performed following the reagent's manufacturer suggested protocol and the total blank was the sum of sample and the reagent blanks.

Weight

For each of the four dietary groups, 10 *Drosophila* adults (5 males and 5 females) were obtained. The flies were rinsed with distilled water and blot-dried using paper towels. Afterwards, collected flies of each sex were weighed on an Adventurer Pro OHAUS analytical balance (precision: 0.0001 g).

Oxidative stress resistance

Newly emerged adult *Drosophila* were obtained and placed in vials containing each of the four experimental diets. After a 6-day aging period, 10 adult *Drosophila*

from each of the vials (5 females, 5 males) were collected and placed in new vials containing a 2.0% agar matrix. The 1.5% hydrogen peroxide matrix contained 2% w/v agar and 5% w/v sucrose in water. Upon initially being agitated by a magnetic stirrer, 12 mL of this solution was added to each culture vial; the 10 adult *Drosophila* were transferred to these vials once the agar solutions solidified. Afterwards, the vials were passed twice daily in order to determine the number of deceased adult *Drosophila*.

Statistical analysis

Results are expressed as mean ± SEM. GraphPad Prism 7 software was used to carry out any statistical analysis, including unpaired t-tests, ANOVAs, Post-hoc tests, and log-rank (Mantel-Cox) tests.

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