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Effect of natural and artificial sweeteners on the hemolymph glucose level (HGL) in *Drosophila melanogaster*

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Abstract

Natural sweeteners are used by consumers to enhance the flavor of food while artificial sweeteners were developed for the same purpose with the added benefit of minimizing the risk of hyperglycemia. Fasting HGL (hemolymph glucose level) of *Drosophila melanogaster* were measured and followed a normal curve distribution. Female flies fasted for 8 hours were fed with reagent-grade and consumer-grade sweeteners and their hemolymph was collected after 1 hour for glucose determination. There was no significant difference in HGL between fasted flies and the flies fed with artificial sweeteners (aspartame, acesulfame-K, saccharin, and sucralose). Flies fed with natural sweeteners increased their HGL in the following order: white sugar > light brown sugar > dark brown sugar > muscovado sugar > coconut sugar > stevia = fasting level. Except for stevia, all the tested natural sweeteners significantly increased HGL compared to fasting HGL ($P < 0.05$). Our study shows that among the natural sweeteners, coconut- and stevia-derived sweeteners, along with muscovado sugar, do not increase HGL as much as the white and brown varieties of cane sugars in *D. melanogaster*. This is the first report to test the effects of an extensive list of sweeteners on circulating glucose levels in a single experimental organism, unlike previous reports. Our findings reveal the suitability of natural sweeteners stevia, coconut sugar, and muscovado sugar as healthier substitutes for white sugar, and may be beneficial for individuals on low calorie diets or those with, or at risk of, hyperglycemia or diabetes mellitus.

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Introduction

Sweeteners have been used for centuries to enhance the flavor of food and to be used as the main ingredients themselves in delicacies. Sucrose from the sugarcane plant of the genus *Saccharum* has been the staple sweetener used worldwide and is commonly called table sugar (International Sugar Organization, 2012). Table sugar is available in white and different brown varieties, depending on the extent of refining and molasses content (Cole, 1939). Traditionally, indigenous groups have produced brown sugar before the advent of industrial means of refining sugar which is used now to produce white sugar (Knight, 2009). However, high sugar diets have been linked to health issues such as dental caries (American Academy of Pediatric Dentistry, 2011), obesity (Morenga and Mallard, 2012), insulin resistance and hyperglycemia (Commerford *et al.*, 2001), type 2 diabetes mellitus (Malik *et al.*, 2010), and even hypertension (DiNicolantonio and Lucan, 2014; Morenga *et al.*, 2014).

Because of people's innate preference for sweetness, various artificial sweeteners have also been synthesized including aspartame, acesulfame-K, saccharin, and sucralose. Also, industrially manufactured sugar alcohols have been added to artificial sweeteners to refine their taste and to serve as bulking agents. The benefits of substituting artificial sweeteners and sugar alcohols for natural sugars include lower calorie intake, lower incidence of dental caries (Hayes, 2001), better glycemic control (Fitch *et al.*, 2012), and weight loss (Miller and Perez, 2014). Despite their availability in the market and being FDA-approved (American Diabetes Association, 2011), potential health concerns on artificial sweeteners have also been raised. Sugar alcohols have laxative effects (Zumbe *et al.*, 2001) while artificial sugars have been implicated in certain cancers (Schernhammer *et al.*, 2012; Yılmaz and Uçar, 2014) and metabolic disorders (Schiffman and Rother, 2013; Swithers, 2013; Araújo, 2014).

This has led to consumers shifting back to traditional sources of sweeteners. In the Philippines, the natural sweeteners muscovado sugar and coconut sugar have found its place in mainstream markets. Muscovado sugar, which is still produced with traditional methods in certain provinces such as Antique and Negros in Central Philippines, is also derived from cane sugar but still retains more molasses compared to brown sugar (Baroña, 2003). Technically referred to as non-centrifugal sugar, it has been the prevailing form of cane sugar in many different countries (called kokuto in Japan, panela in Latin America, jaggery in India) long before industrialization in the 18th century (Jaffé, 2012). Coconut sugar, made from the sap of *Cocos nucifera*, has been widely used in traditional cooking in the Philippines, Indonesia, and Thailand (Purnomo, 2007) and is of great economic importance for local producers (Manohar, 2012). Coconut sugar is classified as a low glycemic index food (Trinidad *et al.*, 2010) and, together with muscovado sugar, has *in vitro* antidiabetic effects (Ranilla *et al.*, 2008). Fig. 1 shows the appearance of white sugars, light brown sugar, dark brown sugar, muscovado sugar, and coconut sugar.

Another sweetener that has been marketed and consumed globally is stevia, a natural sweetener which has been traditionally extracted from *Stevia rebaudiana* by Guarani Indians of Paraguayan long before stevia was introduced in Europe in the 19th century (Brandle *et al.*, 1998). It has been shown to reduce postprandial blood glucose levels (Gregersena *et al.*, 2004), has no side effects (Barriocanal *et al.*, 2008), and does not affect food intake and satiety (Anton *et al.*, 2010). However, the health effects, particularly on glucose levels, of natural sweeteners such as muscovado sugar and coconut palm sugar have not yet been examined.

Drosophila melanogaster has been an important model organism for studying molecular mechanisms of human processes such as metabolism (Bharucha, 2009). Conserved glucose homeostasis mechanisms in *Drosophila melanogaster* and vertebrates include glucose transporters (Ceddia *et al.*, 2003; Baker and Thummel, 2007; Musselman *et al.*, 2011; Na *et al.*, 2013), adipokinetic hormone receptor which act like

glucagon receptors (Bharucha *et al.*, 2008), and insulin-like peptides (Zhang *et al.*, 2009). In this study, we used *Drosophila melanogaster* as an experimental system to directly compare the effects on HGL of an extensive list of reagent-grade and consumer-grade (i.e. store-bought) natural and artificial sweeteners.

Materials and methods

Reagents

Amplex® Red Glucose/Glucose Oxidase Assay Kit (Life Technologies, Grand Island, NY, USA) was used in determining glucose level concentrations. Reagent-grade sweeteners used include sucrose (Sigma-Aldrich, St. Louis, MO, USA), fructose (Sigma-Aldrich, St. Louis, MO, USA), aspartame (Supelco, Bellefonte, PA, USA), saccharin (Sigma-Aldrich, St. Louis, MO, USA), sucralose (Fluka, Sigma-Aldrich, St. Louis, MO, USA), mannitol (Supelco, Bellefonte, PA USA), and xylitol (Sigma-Aldrich, St. Louis, MO, USA). Consumer-grade sweeteners were also used. Deionized water was used in reaction buffer preparations. Carbon dioxide was used in sedating the flies for sorting and ease of handling.

Fly culture and maintenance

Wild type *Drosophila melanogaster* (Oregon-R strain) were obtained from the Department of Biology, University of the Philippines – Baguio. The flies were allowed to reproduce at room temperature on a sweet potato medium with the formulation: 1 L distilled water, 500 g orange sweet potato, 15.4 g agar, 10 g yeast, and 0.08% propionic acid (added after boiled media was cooled to 70°C). Medium was placed in sterile bottles. A small amount of yeast paste, made with active dry yeast in 0.08% propionic acid, was also added as a protein source for the flies.

New (~1 day old) flies from hatched pupae in a bottle were immediately transferred to two separate bottles containing freshly prepared sweet potato medium. These flies were allowed to mate and reproduce for five days before they were transferred again to another set of bottles containing freshly prepared sweet potato medium. For age control, flies were emptied out of bottles containing pupae. After a day, newly emerged flies were isolated and were labeled one-day old flies. For sex control, flies were sedated with carbon dioxide on a CO_2 pad under a stereoscope. Females were segregated from males within five minutes using a fine paintbrush and were transferred into a vial with fresh medium.

Base curve of fasting HGL of Drosophila

Five-day old female flies (Oregon-R strain) were starved for 8 hours in a bottle without medium. The flies were then transferred to a bottle containing filter paper saturated with sterile distilled water. An hour after hydration, the flies were sedated with carbon dioxide and once unconscious, they were immobilized on their sides on a glass slide with two-sided adhesive tape. The head of the fly was punctured with a glass microneedle at the mid-dorsal area and the abdomen of the fly was gently pressed to allow hemolymph to extrude from the punctured head. A filter paper disk was used to absorb a total 0.5 µL of extruded hemolymph from a pool of 20 to 30 flies. The disk was resuspended in 149.5 µL of the reaction buffer. The solution was centrifuged for 10 minutes at 13,000 X g to precipitate tissue debris and blood cells. 50 µL of the supernatant was assayed for glucose concentrations, following the protocol provided by the Amplex® Red Glucose/Glucose Oxidase Assay Kit. The process was repeated until a total of 50 hemolymph pooled samples were taken. The data was statistically analyzed and a base curve for the fasting glucose level concentration in *Drosophila melanogaster* hemolymph was generated using Microsoft Excel.

Effect of reagent-grade sweeteners on HGL

Five-day old female flies were starved for 8 hours. The flies were then transferred into separate bottles and were fed with different sweeteners. For the effect of the sweeteners on glucose level based on equal molarities, flies were fed with 60.00 mM of various sweetener solution saturated on a filter paper. For the effect of the sweeteners on glucose level based on equal level of sweetness, solutions of sucrose (60.00 mM), fructose (42.90 mM), saccharin (0.133 mM),

aspartame (0.279 mM), sucralose (0.100 mM), xylitol (60.00 mM), and mannitol (100.00 mM) were given to the flies. Equisweet concentrations were computed using the following equation (where EqsC = equisweet concentration, C_{suc} = concentration of sucrose reference, and SF_{suc} = sweetness factor relative to sucrose as listed in Table 1):

$$EqsC = \frac{C_{suc}}{SF_{suc}}$$

An hour after feeding, flies were sedated, hemolymph was collected, and glucose was determined as described in the generation of fasting HGL base curve above. A total of 5 pools of hemolymph was collected and analyzed. The percent decrease in HGL of the sweeteners with respect to sucrose ($\%dec_{wrts}$) was computed as follows (HGL_s = HGL after sucrose feeding, HGL_f = fasting HGL, HGL_x = HGL after sweetener feeding):

$$\% \, dec_{wrts} = \frac{(HGL_s - HGL_f) - (HGL_x - HGL_f)}{(HGL_s - HGL_f)} \times 100$$

Comparison of mean HGL of each treatment and mean fasting HGL were performed using ANOVA and t-test (α = 0.05).

Effect of consumer-grade sweeteners on HGL

Five-day old female flies were starved for 8 hours. Flies were then transferred into separate bottles and were fed with sweetener solutions saturated on a filter paper. Based on equal weights, 0.103 g each of the sweeteners was dissolved in 5 mL sterile distilled water. For its effect based on equal level of sweetness, sweeteners were dissolved in 5 mL of sterile distilled water: white sugar (0.103 g), coconut sugar (0.036 g), stevia (0.007 g), aspartame (0.010 g), acesulfame-K (0.010 g), saccharin (0.008 g), sucralose (0.010 g), sorbitol (0.026 g), and xylitol (0.015 g). For the effect per serving of commercial sweeteners, solutions were prepared by dissolving suggested serving size of sweeteners to 5 mL of sterile distilled water: white sugar (0.103 g), light brown sugar (0.103 g), dark brown sugar (0.103 g), muscovado sugar (0.103 g), coconut palm sugar (0.051 g), stevia (0.015 g), aspartame (0.015 g), acesulfame-K (0.015 g), saccharin (0.012 g), sucralose (0.015 g), sorbitol (0.037 g), and xylitol (0.022 g). An hour after feeding, flies were sedated, hemolymph was collected, and glucose was determined as described in the generation of fasting HGL base curve. A total of 5 pools of hemolymph was collected and analyzed. Equisweet concentrations were computed using the following equation (where EqsC = equisweet concentration, $C_{white\ sugar}$ = concentration of white sugar reference, and $SF_{white\ sugar}$ = sweetness factor relative to sucrose as listed in Table 2, and FPS = fraction of the sweetener in the store-bought package):

$$EqsC = \frac{C_{white\ sugar}}{SF_{white\ sugar}} \times \frac{1}{FPS}$$

The percent decrease in HGL of the sweeteners with respect to white sugar was computed as described in the effect of reagent-grade sweeteners on HGL. Comparison of mean HGL of each treatment and mean fasting HGL were performed using ANOVA and t-test (α = 0.05).

Results and discussion

Fasting HGL of Drosophila melanogaster (Oregon-R)

The HGL of female *Drosophila melanogaster* (Oregon-R strain) was measured using Amplex® Red Glucose/Glucose Oxidase Assay Kit and was found to have a normal distribution with a mean of 17.812 ± 2.191 mM and with skewness of 0.070 as seen in Fig. 2. This mean fasting HGL lies within the range of those measured in previous studies wherein the fasting HGL in female *white*^{Dahomey} flies was approximately 2.2 mM (Broughton *et al.*, 2005), in adult *white*^{Dahomey} flies was approximately 10 mM (Broughton *et al.*, 2008), and in 14-day old adult *white*^{1118} flies was approximately 30.5 mM (Haselton *et al.*, 2010). All these previous studies measured HGL using Infinity™ Glucose reagent. The differences in the measured fasting HGL among these studies may be attributed to differences in the strain

of the flies used, the sex of flies tested, the duration of starvation, and the diet of the flies before fasting HGL was determined. In spite of the apparent variability in the fasting HGL of *Drosophila melanogaster*, these reported fasting HGL values are all valid in their own experimental parameters.

Table 1. Sweetness factors of reagent-grade sweeteners relative to sucrose.

Sweetener	Sweetness factor relative to sucrose (sucrose = 1.0)	Reference
fructose	1.4	NPCS Board (2012)
saccharin	450	Bettelheim *et al.* (2010)
aspartame	215	Inglett (1981)
sucralose	600	Fitch *et al.* (2012)
xylitol	1.0	Fitch *et al.* (2012)
mannitol	0.6	Fitch *et al.* (2012)

Reagent-grade sweeteners on HGL

The effects of reagent-grade sweeteners on *D. melanogaster* HGL were determined for solutions with equal molarity (Fig. 3A) and equal levels of sweetness (Fig. 3B). The natural sugars, sucrose and fructose, significantly increased HGL compared to fasting levels ($P < 0.05$). However, HGL of fructose-fed flies was at least 70% lower compared to sucrose-fed flies for both equimolar and equisweet preparations.

Table 2. Sweetness factors of consumer-grade sweeteners relative to white sugar.

Sweetener	Sweetness relative to white sugar[a] (white sugar = 1)
coconut sugar	2.9
stevia	14.7
aspartame	10.3
acesulfame-K	10.3
saccharin	12.9
sucralose	10.3
sorbitol	4.0
xylitol	6.8

[a]These sweetness factors were computed from the information indicated on the packaging of each store-bought sweetener.

We think that the increase in HGL in *D. melanogaster* was due to the hydrolysis of the disaccharide sucrose into glucose and fructose units. Additionally, these fructose units may be converted to glucose *via* gluconeogenesis. The enzymes for sucrose hydrolysis and gluconeogenesis are present in -*Drosophila* (Marzluf, 1969; Flowers *et al.*, 2007). Consistent with other reports, a diet high in sucrose increases HGL and may lead to hyperglycemia in wild type *D. melanogaster* (*Canton-S*) larvae (Musselman *et al.*, 2011). Fructose, on the other hand, has a lower glycemic index in adult flies compared to glucose (Rovenko *et al.*, 2015). All these observations in *D. melanogaster* are also seen in human studies. A previous study demonstrated that in normal humans, sucrose has a high glycemic index while fructose has a low glycemic index (Wolever, 1998).

This is consistent with another study that showed that the increase in blood glucose level in humans is significantly lower after ingestion of fructose compared to sucrose (Lee, 1997).

Whether equimolar (Fig. 3A) and equisweet (Fig. 3B) solutions were fed to *Drosophila*, artificial sweeteners

did not significantly increase HGL compared to the fasting levels. This is expected as aspartame, saccharin, and sucralose are non-nutritive and are not converted to glucose. Hence, HGL is insensitive to changes in concentrations of these artificial sweeteners. This response to artificial sweeteners is similar to humans (Fitch *et al.*, 2012) and thus validates the use of *Drosophila* as model of human glucose homeostasis.

Fig. 1. Images of natural sweeteners produced in the Philippines: (A) white sugar, (B) light brown sugar, (C) dark brown sugar, (D) muscovado sugar, (E) coconut sugar, and (F) stevia. A to F are derived from sugarcane, E is derived from coconut, and F is derived from stevia plant.

Mannitol, similar to artificial sugars, did not affect HGL (Fig. 3A and 3B). Similarly, mannitol does not affect blood glucose levels in humans (Olmsted, 1953). Xylitol increased HGL but was at least 80% lower compared to sucrose for both equimolar and equisweet solutions. Xylitol enters the pentose phosphate pathway *via* xylulose-5-phosphate (Brunzell, 1978) and may lead to glucose production *via* gluconeogenesis. In *Drosophila*, gluconeogenesis and pentose phosphate pathway have been characterized (Ceddia *et al.*, 2003; Xie *et al.*, 2013). Consistent with human studies, xylitol has low glycemic index and elicits a small insulin response (Natah *et al.*, 1997).

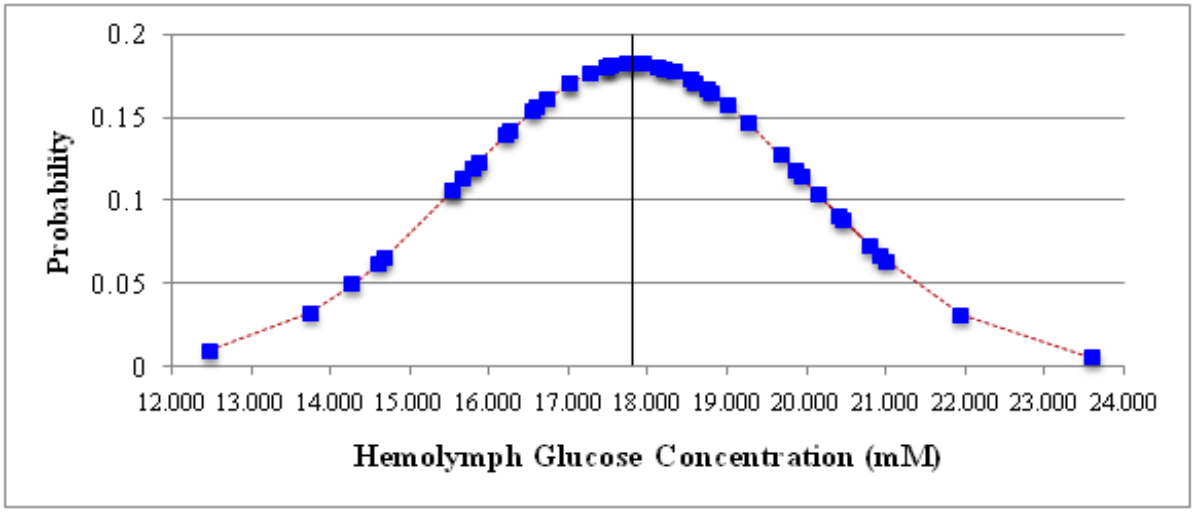

Fig. 2. Normal curve for the fasting glucose level concentration in *Drosophila melanogaster* hemolymph (n = 50). Mean HGL: 17.812±2.191 mM.

It is notable that the equimolar and equisweet preparations of the solutions yield similar trends and similar values in *D. melanogaster* HGL, except for fructose-fed. The HGL of fructose-fed flies had a small drop in glucose levels proportional to the difference in equimolar and equisweet concentrations of the fructose solutions. These results indicate that HGL is sensitive to reagent-grade preparations of natural sugars but insensitive to artificial sugars in *D. melanogaster*.

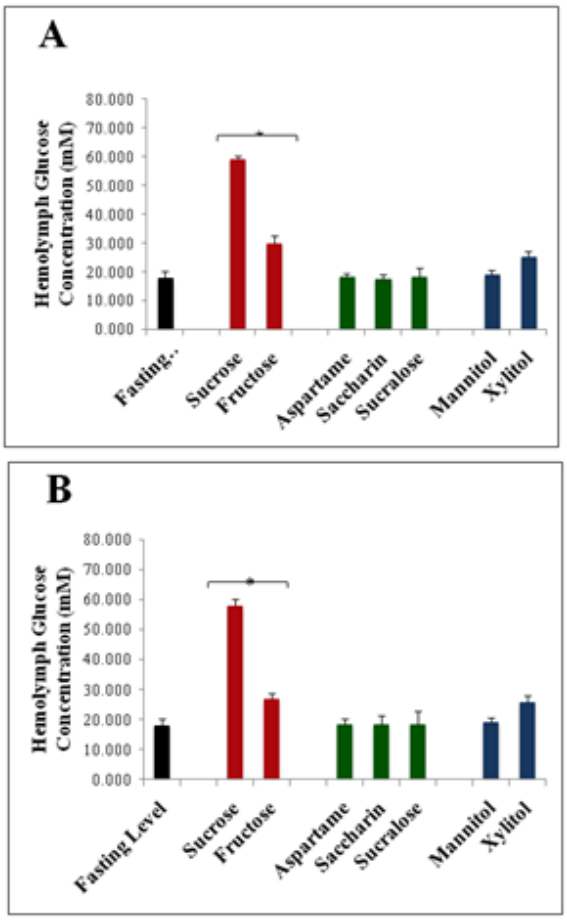

Fig. 3. Effect of reagent-grade natural sweeteners (red), artificial sweeteners (green), and sugar alcohols (blue) on *D. melanogaster* HGL (n = 5). Solutions were prepared with (A) equal molarities and (B) equal levels of sweetness. * $P < 0.05$ with respect to fasting HGL.

Consumer-grade sweeteners on HGL

The effects of consumer-grade sweeteners on HGL were determined for solutions with equal weights (Fig. 4A), with equal levels of sweetness (Fig. 4B), and with suggested servings (Fig. 4C). The suggested serving is of practical importance as it is the amount packaged by manufactures and commonly used by consumers. It was assumed that the suggested serving for each sweetener has been measured to match the sweetness of a serving of white sugar.

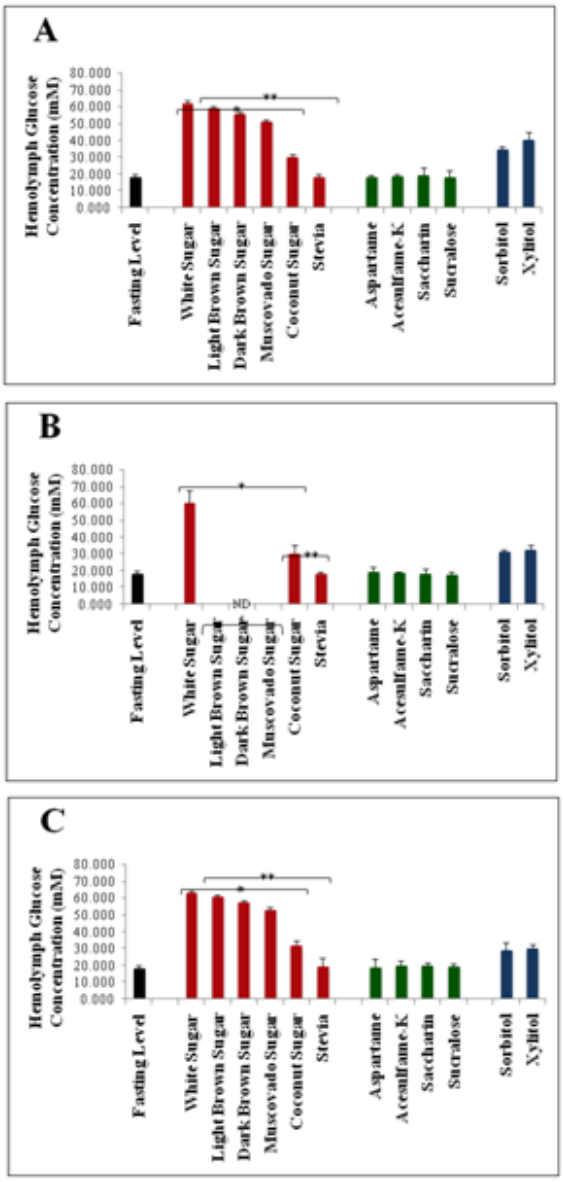

Fig. 4. Effect of consumer-grade natural sweeteners (red), artificial sweeteners (green), and sugar alcohols (blue) on *D. melanogaster* HGL (n = 5). Solutions were prepared with (A) equal weights, (B) equal levels of sweetness (ND = not determined), and (C) suggested servings. * $P < 0.05$ with respect to fasting HGL.

** $P < 0.05$ with respect to white sugar.

As seen in the different sweetener preparations in Fig. 4 (A, B, and C), the spikes in HGL relative to fasting levels were consistent: white sugar > light brown

sugar > dark brown sugar > muscovado sugar > coconut sugar > stevia = fasting HGL. Stevia is the most remarkable among the natural sweeteners tested as it did not increase HGL compared to fasting levels (P > 0.05). The abundant flavor principles of stevia – stevioside and rebaudioside A – are not saccharides but they still bind to the human heteromeric sweet taste receptors, hTAS1R2/hTAS1R3, which are able to respond to chemically diverse sweeteners (Hellfritsch *et al.*, 2012). Although these molecules are attached to glucose moieties in stevia extract, they have been shown to mimic insulin by modulating glucose transport *via* the PI3K/Akt pathway (Rizzo *et al.*, 2013). Being more than 200 times sweeter than sucrose (Hellfritsch *et al.*, 2012) without affecting circulating glucose levels, stevia is very suitable as a substitute for table sugar.

Coconut sugar is notable as *D. melanogaster* HGL was at least 69% lower when fed with this sweetener compared to white sugar. Although coconut sugar contains 70-79% sucrose, 3-9% glucose, and 2-9% fructose as reported previously (Purnomo, 1992), it does not increase HGL in flies as much as refined sugar. Thus, we conclude that stevia and coconut sugar, which are not from sugarcane, do not raise HGL as much as the sugarcane-derived ones in *D. melanogaster*.

Among cane sugars, there is a trend of higher HGL as the sugar gets more refined. HGL of flies fed with muscovado sugar, the least refined cane sugar tested, was at least 22% lower compared to those fed with white sugar. Various studies have reported the health benefits of muscovado sugar, particularly antidiabetic effects (Jaffé, 2012). Other potential benefits include high phenolic content and DPPH radical scavenging activity in muscovado sugar from Mauritius (Ranilla *et al.*, 2008). In the same study, brown sugars fared better in these antioxidant measures compared to white sugar.

Similar to reagent-grade artificial sweeteners, the consumer-grade artificial sweeteners did not significantly change HGL compared to fasting levels (P > 0.05). On the other hand, the sugar alcohols sorbitol and xylitol increased HGLs proportional to their concentration (across preparations of equal weight, equal sweetness, and suggested serving). HGL was 76% lower for sorbitol-fed flies and 73% lower for xylitol-fed flies compared to the sucrose-fed flies when the sweetener solutions were prepared based on their suggested servings. Sorbitol dehydrogenase, which catalyzes the conversion of sorbitol to fructose, has been characterized in *Drosophila* (Luque *et al.*, 1998). The fructose derived from sorbitol can lead to glucose production *via* gluconeogenesis (Chandramouli *et al.*, 1993), just like in xylitol (Brunzell, 1978).

Our findings reveal the suitability of natural sweeteners stevia, coconut sugar, and muscovado sugar as healthier substitutes for refined white sugar, in addition to the usual artificial alternatives like saccharin, aspartame, and sucralose. This is important for individuals with caloric restrictions and in patients with hyperglycemia or diabetes mellitus. Consumers will be more receptive to natural sweeteners than artificial sweeteners because the former is not hampered with unresolved health issues and concerns unlike the latter.

Despite being displaced by refined white sugar, natural sweeteners have re-emerged in the global market today because consumers search for organic and healthier alternatives. The resurgence of natural sweeteners could impact the small players of the sugar industry in rural locales. As production of natural sugars are less costly and need no sophisticated machinery, community-based producers can compete in the sugar industry. Moreover, as stevia is already widely marketed, investors can expect an additional demand for this sweetener and can even establish local industries for its production.

In the past, it was difficult to directly compare several sweeteners with regard to their effects on circulating glucose levels because previous studies used different

test systems and evaluated few sweeteners at a time. To our knowledge, this is the first report to test an extensive list of sweeteners in a single experimental system (HGL in *Drosophila melanogaster*), allowing direct comparison between a greater number of sweeteners.

Conclusion

The re-emergence of natural sweeteners today benefits both consumers and producers. Consumers of natural sweeteners now have healthier options to replace table sugar. On the other hand, producers of natural sweeteners, being small players in the global sugar industry, have a market to sell their local produce. Our study has shown that the natural sweetener stevia and coconut sugar do not increase HGL as much as white sugar (table sugar) in *D. melanogaster*. Additionally, muscovado sugar, the least refined and noticeably the darkest variety of the cane sugars, resulted in the lowest HGL compared to the other lighter-colored varieties of cane sugar. Moreover, our study adds to the growing body of evidence that *Drosophila melanogaster* is a valid test organism to model human glucose metabolism. We therefore recommend using *Drosophila* in assessing other existing and potential sweeteners.

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Authors' Contributions

Conceived and designed the experiments: PMM. Performed the experiments: JSC PMM. Analyzed the data: JSC PMM JLA. Wrote the paper: PMM JLA.

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