A novel method for measuring fructose ingestion by mosquitoes

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ABSTRACT: We adapted the Seliwanoff method to quantify fructose in mosquitoes. This method showed a minimum detection limit of 2.4 µg of fructose, and was more reliable and nearly four times more sensitive than the anthrone test. The Seliwanoff method was used to measure the maximum sugar intake by individual mosquitoes and to determine the digestion time of this nutrient by both Aedes aegypti and Aedes albopictus in the laboratory. Sugar intake by Ae. albopictus was up to 1.7 times higher than that of Ae. aegypti. The amount of sugar ingested by females was up to 2.5 times higher than that of males in both species. After 48 h, a fructose meal was not detected any longer in either species. The Seliwanoff method was applied to measure fructose content of field-collected Ae. aegypti males and females in Rio de Janeiro. Results showed that even Ae. aegypti females do feed on sugars. The standardized Seliwanoff method proved to be reliable for measuring the sugar content of individual mosquitoes and can be used wherever estimation of small quantities of fructose is needed. Journal of Vector Ecology 33 (2): 225-231. 2008.

Keyword Index: Aedes aegypti, Aedes albopictus, fructose, sugar feeding, metabolism.

INTRODUCTION

The information available on energy metabolism of mosquitoes is far less than adequate. It is generally accepted that, like other mosquitoes, Aedes aegypti and Aedes albopictus feed on fruit juice and flower nectar to obtain carbohydrates, which are used as energy sources both for flight and also for several other metabolic demands (Foster 1995, Briegel et al. 2001). These species are anautogenous, meaning that females must feed on blood in order to produce eggs. The amount of blood ingested by mosquito females has been correlated to the number of eggs laid (Briegel 1990a, 1990b, Clements 1992, Takken et al. 1998, Roitberg et al. 2005). Of indisputable relevance however, is the fact that feeding on sugar increases the reproductive success of these species, because it enhances egg production (Nayar and Sauerman 1975, Gary and Foster 2004, Zhou et al. 2004a, 2004b).

Sugar content of field-collected mosquito specimens has been measured in order to evaluate the effect of sugar ingestion on their vector capacity, (Edman et al. 1992, Van Handel et al. 1994, Martinez-Ibarra et al. 1997, Costero et al. 2004a, 2004b). These studies surprisingly concluded that Ae. aegypti females rarely feed on sugar in nature. Instead, these authors claimed that wild Ae. aegypti females use blood feeding as an almost exclusive food source. These findings contradict the generally accepted idea that mosquito females feed both on sugar and on blood and that this behavior increases their reproductive success (Ziegler 1996, Clements 1992, Yuval 1992, Foster 1995, Mostowy and Foster 2004, Zhou et al. 2004a, 2004b). Do Ae. aegypti females indeed rarely or never take sugar meals in nature? Alternatively, the most commonly used method for sugar detection and quantifying in mosquitoes – the anthrone test – is not as sensitive as expected and may produce false negatives.

In this paper we standardized a method for the detection of sugar intake by Ae. aegypti and Ae. albopictus, comparing its performance with that of a previously used method, the anthrone test, and measured fructose content of Ae. aegypti collected in a dengue endemic area, in Nova Iguaçu, Brazil.

MATERIALS AND METHODS

Mosquitoes

Wild Aedes aegypti collected from Rosa dos Ventos neighborhood, an urban area of Nova Iguaçu, state of Rio de Janeiro, Brazil, as well as laboratory-reared specimens of Ae. aegypti (Rockefeller strain) and Aedes albopictus (a colony that originated with wild-caught mosquitoes from Rio de Janeiro) were used in this investigation. Larvae were maintained on a diet consisting of dog chow (Consoli and Lourenço-de-Oliveira, 1994). Adults were kept at 28°C, 80% relative humidity, a photoperiod of 12:12 (L:D) and allowed ad libitum access to 10% sucrose solution until one to two days before the experimental procedures.
Laboratory sample preparation

Laboratory-reared mosquitoes were starved for 24 h and then allowed to feed for one hour on 10% blue aniline stained sucrose solution. Aniline staining was used to make it easier to recognize mosquitoes that ingested the sugar solution. Engorged mosquitoes were separated by sex into groups of 60 individuals and killed by freezing (-20° C), either immediately or 12 h and 24 h after sugar ingestion. Whole mosquitoes were manually and individually homogenized in 150 µl of distilled water. The liquid fraction was transferred to a 1.5 ml tube and centrifuged for 10 min at 13,000 g. Finally, fructose content from an aliquot of the supernatant (100 µl – 2/3 of the mosquito homogenate) was measured and the sugar content of each mosquito was calculated. It was assumed that the sucrose ingested by the mosquitoes would be hydrolyzed to glucose and fructose, either by the mosquito enzymes or during the chemical quantification of the sugars carried out in strongly acid pH.

Anthrone test

The reagent was prepared by adding 25 mg of anthrone to 50 ml of 70% sulfuric acid, previously diluted and cooled (Van Handel 1985, Costero et al. 1998). A standard curve was obtained by using from 2 to 35 µg of fructose in 25% ethanol in the reaction. The minimum limit for sugar detection considered to be reliable was the one that gave an absorbance of 0.1.

The reaction was carried out for 75 min at room temperature (~25° C) after the addition of 100 µl of sample to 3 ml of the reagent, and read at 625 nm in a spectrophotometer (Ultra Spec Pharmacia, Biotech).

Seliwanoff test

The reagent was prepared by adding 0.1 g of resorcinol to 200 ml of 6N chloridric acid (Stanek et al. 1963). The Seliwanoff major reagent, resorcinol, reacts with ketoses, forming a red compound with a maximum absorption at 473 nm.

A standard curve was obtained using from 2 to 40 µg of fructose. The minimum limit for sugar detection considered to be reliable was the one that gave an absorbance of 0.1. The reaction was carried out for 3 min in a boiling water bath, after the addition of 100 µl of sample to 500 µl of the reagent. The reaction mixture was centrifuged and an aliquot of 500 µl of the supernatant was read at 484 nm in a spectrophotometer.

Thin layer chromatography (TLC)

Standard samples of fructose, sucrose, glucose, trehalose, and laboratory-reared mosquito centrifuged homogenates were submitted to a 12 h ascendant thin layer chromatography using a mixture of n-propanol: benzyl alcohol: formic acid (85%): distilled water (12:18:5:5) as solvent. The thin layer chromatography plate used was Merck Silica gel 60. The Seliwanoff reagent was used as staining reagent. The reaction took place inside a warm oven-like chamber at 100° C for 3 min.

Statistical analysis

Results were analyzed using One Way ANOVA with post-test of Bonferroni. Geometric means were considered different when a statistical significance level (α) lower than 0.05 was found. All the plots and analysis were performed using Graph Pad Prism 3 software.

RESULTS

The fructose standard curve for the anthrone reagent (inset in Figure 1) was fairly linear (R² = 0.9955). The minimum detection limit was 10 µg of fructose, which is a value higher than what is expected for an individual mosquito measurement. The standard curve for the reaction of fructose with the Seliwanoff reagent (Figure 1) was also linear (R² = 0.9941), but this method allowed the detection
of lower amounts of fructose: 2.4 µg of fructose (nearly four times more sensitive than the anthrone test). Besides being more sensitive, the Seliwanoff method was capable of reliably measuring fructose even in the presence of glucose and other potentially interfering substances present in the crude mosquito homogenates. This can be seen as a result of the thin layer chromatography exhibited on Figure 2. In mosquito samples the only spots revealed on the plate are those corresponding to fructose derived from sucrose hydrolysis by the staining reagent (same migration in lanes 2 – standard sucrose – 3 and 5, mosquito samples) and to fructose derived from the sucrose ingested and hydrolyzed to its monosaccharide components, probably by mosquito enzymes (respectively lanes 3 and 5). Mosquito samples should be compared to lane 4, since fructose migration is somewhat altered when it is in a mixture.

Fructose content was individually quantified in laboratory-reared mosquitoes in both species soon after feeding with sugar as well as 12 and 24 h after a sugar meal (Figures 3A and 3B). The amount of sugar ingested by females was much higher than that of the males of same species: 1.6 times for Ae. aegypti (p=0.01) and 2.5 times for Ae. albopictus (p=0.001). Comparing the sugar intake by female mosquitoes: sugar intake by Ae. albopictus was much higher than that of Ae. aegypti (1.7 times, p=0.001). The amount of sugar intake by males of both species was similar.

Ae. aegypti and Ae. albopictus males and females from laboratory colonies showed the same pattern of sugar digestion. There was a great decrease in fructose content in the first 24 h of fasting and a small decrease in the following 24 h period, except for Ae. albopictus females (Figures 3A and 3B), which seem to digest sugar much more slowly.

Although these mosquito species showed the same time pattern of sugar digestion, there is a significant difference in the sugar consumption profiles of laboratory-reared females and males of both species (Figure 4, Table 1): Ae. aegypti males digest about 30% of the ingested sugar in 12 h, whereas Ae. aegypti females digest up to 70% of the ingested sugar in the same 12 h period and only 10% in the following 12 h. Ae. albopictus males consume 55% of the sugar intake in the first 12 h and 30% in the following 12 h, whereas Ae. albopictus females consume only 5% in the first 12 h and 35% in the following 12 h.

The mean fructose content of field-collected Ae. aegypti males, measured using the Seliwanoff method, was twice that of females collected in the same day (Figure 5), even though the mean sugar content of Ae. aegypti females was slightly higher than the cut-off value. Several wild females have sugar content high enough to assure that sugar intake has occurred in the last 24 h. The percentage of male Ae. aegypti that show sugar level compatible with recent sugar intake (positive) was 47.6%, nearly two times higher than that of females (27.1%). Mosquitoes collected on different days showed significant differences in sugar content.

DISCUSSION

Sugar is the basic food for adult mosquitoes, but sugar feeding by female Ae. aegypti in nature is under debate (Foster 1995, Ziegler and Ibrahim 2001). In his pioneer work, Van Handel (1972) used the anthrone test to quantify sugars in mosquitoes by preparing homogenates of ten individuals in order to recover enough sugar to be detected by this method, for which the lower detection limit is 10 µg of sugar. Our results confirmed the sensitivity level observed by Van Handel. The anthrone test is not sensitive enough to detect fructose concentrations below 10 µg per test using 3 ml of the reagent.

We performed the anthrone test in homogenates

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Hours after sugar ingestion (min.)</th>
<th>Fructose (µg) per individual</th>
<th>p value</th>
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</thead>
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<tr>
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</tr>
<tr>
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<tr>
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<td>Aedes albopictus</td>
<td>Female</td>
<td>24</td>
<td>19.1</td>
<td></td>
</tr>
</tbody>
</table>

Table I: Fructose content of Ae. aegypti and Ae. albopictus. The values presented were obtained calculating the geometric means of 60 individual mosquitoes in each condition.
Figure 2. Twelve h ascendant thin layer chromatography on Merck Silica gel 60, using a mixture of n-propanol: benzyl alcohol: formic acid (85%): distilled water (12:18:5:5) as solvent. Seliwanoff was used as staining reagent; the reaction was developed in a warm chamber at 100°C by 3 min. 1 – Trehalose, 2 – Sucrose, 3 – Extract from two Ae. aegypti females obtained immediately after feeding, 4 – Glucose + Fructose + Sucrose + Trehalose, 5 – Extract from five Ae. aegypti females obtained 12 h after feeding, 6 – Glucose, 7 – Fructose.

Figure 3. Fructose content of Ae. aegypti (A) and Ae. albopictus (B) at 0, 12, and 24 hours after sucrose ingestion. Each box shows the frequency distribution of fructose content for 60 mosquitoes individually tested with the Seliwanoff method. Light Box – Male; Dark Box – Female.
derived from pools of mosquitoes of several body sizes, aiming to simulate the variability found in field-collected mosquitoes. We observed that when the amount of fructose contained in the sample was lower than 10 µg, the color of the anthrone reaction was equal to that of the blank. Nevertheless, when placed in a spectrophotometer cuvet, these samples returned an absorbance reading, which, in our opinion, was due only to turbidity. To confirm this hypothesis we performed a wavelength scan of samples containing 20, 10, 5, 2 and 1 µg of fructose and submitted to perform the anthrone reaction as described, using an extremely sensitive spectrophotometer (Ultrospec U200 Pharmacia). The chromophore peak was absent when fructose is less than 5 µg (data not shown).

We are confident that the Seliwanoff method is preferable to the most currently used anthrone test to detect and quantify sugar intake by mosquitoes, since the Seliwanoff test can reliably detect 2.4 µg of fructose in a mosquito homogenate, being nearly four times more sensitive than anthrone.

The supernatant of a crude mosquito homogenate is a complex mixture. To ensure that the Seliwanoff reaction with mosquito homogenates was detecting only fructose, those homogenates were fractionated through a thin layer chromatography. The Seliwanoff reagent was used to reveal the chromatogram aimed at detecting any interfering substance in this extract. Although the run for fructose showed interference when in a mixture (lane 4), it can be seen that the only spots revealed in mosquito samples (lanes 3 and 5), were those corresponding to fructose derived from sucrose hydrolysis by the staining reagent and to fructose derived from the ingested sucrose by the insects. An interesting result of this was the detection of fructose almost immediately after sucrose was ingested (1 h after feeding). This indicates that sucrose hydrolysis occurs inside the crop. Further studies are necessary to establish if this hydrolysis is due to the action of mosquito enzymes or to the action of microorganisms present in the crop.

In the reaction conditions described herein, the Seliwanoff reagent does not react with other sugars (glucose and trehalose) that may be present in significant quantities in mosquitoes. This was demonstrated by incubating solutions of each of these sugars with the reagent in the conditions described under methods (data not shown). Even when as much as 100 µg of these sugars were added to the Seliwanoff reagent, no color could be detected after 3 min in boiling water.

Our aim was to standardize a method for the evaluation of sugar intake and sugar digestion time applicable to field-captured mosquitoes in order to assess this feature in Brazilian mosquito populations. For doing that, fructose content was quantified by the Seliwanoff method in individually examined laboratory-reared mosquitoes. Individuals from two Aedes species were examined right after feeding with sugar and 12 and 24 h latter. The amount of sugar ingested by females was much higher than that of the males of the same species, and the quantity of sugar intake by males of both species was similar. Sugar intake in *Ae. albopictus* females was much higher than that of *Ae. aegypti* females. It may be that *Ae. albopictus* is more avid for carbohydrate, regardless of being usually smaller than *Ae. aegypti*. That could be explained in two ways: *Ae. albopictus* are less efficient on using sugar and/or they have more need for sugar, since *Ae. albopictus* females are usually more active when host-seeking. These are hypotheses worth investigating.

![Figure 4. Fructose consumption of males (□) and females (■) of *Ae. aegypti* (A,B,E,F) and *Ae. albopictus* (C,D,G,H) in a 12-h period after sucrose feeding (left panel) and the total consumption in 24 h (right panel). Each bar shows the means of the percentage of fructose consumed by 60 mosquitoes individually tested with the Seliwanoff method.](image-url)
Before feeding our test mosquitoes, we starved them for 24 h to better distinguish engorged mosquitoes from poorly fed ones. As that scenario might not occur in nature, we performed experiments with mosquitoes fed ad libitum in sugar solutions (data not shown). We found that the average amount of sugar content in these non-previously starved individuals was lower than the amount found in the group where feeding was preceded by starving. However, these mosquitoes tended to achieve the same physiological sugar level after 12 h of starvation as the mosquitoes starved for 24 h before the experiment. This observation shows that the starvation period we imposed on mosquitoes in the laboratory does not cause interference in insect metabolism that would prevent the use of the described method for the evaluation of sugar intake in wild mosquitoes. Moreover, these results suggest that, similarly to what occurs with mammals, which keep their sugar blood levels steady upon starvation, mosquitoes manage to keep adequate sugar availability in the hemolymph. We think that there is an integrated fine tuning control of glycogen and trehalose reserves in tissues, hemocele sugar content, and free, still unemployed carbohydrates in the crop.

The Seliwanoff reagent can be used to quantify fructose in mosquitoes in quantities as low as 3.4 µg per individual. Indeed, 3.4 µg of fructose was shown to be the point of Seliwanoff colorimetric change. This detection limit associated with our results allows someone to state that a mosquito collected in the field that is negative for fructose using this method has not feed on sugar for at least 24 h. On the other hand, mosquitoes that did feed on sugar in the last 24 h will test positive for sugar intake. The turbidity caused by lipids could give a false positive for fructose when mosquitoes are grounded mechanically instead of manually. The misinterpretation of a positive absorbance reading can be avoided because the color expected as a consequence of sugar reaction with the Seliwanoff reagent would be absent if there was no sugar present. Lipids did not show colorimetric changes and the detected turbidity was due to the homogenizing process that may have caused lipid emulsification.

When field-collected mosquitoes were examined for previous fructose intake, it was observed that the mean fructose content of *Ae. aegypti* males was higher than that of females collected on the same day. As females also feed on blood, this result was not unexpected. Even though the mean sugar content in *Ae. aegypti* females was only slightly higher than the cut-off level, we are confident that, different from other published data (Costero et al. 1998), *Ae. aegypti* females also feed on sugar in field conditions found in Rio de Janeiro.

The differences found in sugar content of same gender mosquito groups collected on different days may be due to differences in the weather. On the day we found the lower number of individuals positive for sugar content, it had rained on the previous days and the temperature was 22°C with 80% relative humidity. On the day a larger number of positive individuals was found, it rained very little the previous day and the temperature was 26°C with 80% relative humidity. The combination of higher temperature and humidity greatly influences mosquito behavior, and more mosquitoes should be collected in the same day to allow better statistical analyses and comparisons. Nevertheless, these results indicate that in field conditions prevailing in Rio de Janeiro, *Ae. aegypti* females do feed on sugar.

An important concern for the use of any colorimetric method for sugar quantification in wild-caught mosquitoes is the possible interference of a previous blood meal and other sugars in the sugar content determination. Concerning blood interference, we tested 60 mosquitoes and found that no interference was detected when the Seliwanoff method was used (unpublished data). The red hemoglobin molecule that might interfere with absorbance readings at 484 nm is

![Figure 5. Fructose content of each field-collected mosquito. Each symbol represents one mosquito. Δ – Male; Ο – Female.](image-url)
precipitated by heat exposure followed by centrifugation. Any heme molecules released from protein in the sample treating method is of no concern because heme precipitates in acid solutions such as the Seliwanoff reagent (HCl 6N) and its color cannot interfere.

We conclude that the Seliwanoff method can be used in samples of biological origin such as mosquito homogenates whenever estimation of small quantities of fructose is needed. We are also convinced that the prior conclusion that "Ae. aegypti females, in nature, seldom feed on sugar" (Costero et al. 1998) is not correct.

REFERENCES CITED

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