An Investigation into the Effects of Homopteran Honeydew Sugars versus Floral Nectar Sugars on Black Fly Longevity, Flight Performance and Digestion

by

Trudy K. Stanfield, B.Sc.

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General Introduction

Floral nectar is thought to be the primary carbohydrate source for most dipteran species. However, it has been shown that black flies (Burgin & Hunter 1997 a,b,c), mosquitoes (Foster 1995; Burkett et al. 1999; Russell & Hunter 2002), deer flies (Magnarelli & Burger 1984; Janzen & Hunter 1998; Ossowski & Hunter 2000), horse flies (Schutz & Gaugler 1989; Hunter & Ossowski 1999) and sand flies (MacVicker et al. 1990; Wallbanks et al. 1990; Cameron et al. 1992, 1995; Schlein & Jacobson 1994, 1999; Hamilton & El Naiem 2000) feed on homopteran honeydew as well as floral nectar.

Prior to 1997 floral nectar was thought to be the main source of carbohydrates for black flies. However, Burgin & Hunter (1997a) demonstrated that up to 35% of black flies had recently consumed meals of homopteran honeydew. This information has necessitated a re-assessment of many life history aspects of black flies. Attempts are being made to examine the effects of nectar versus honeydew on black fly fecundity and parasite transmission (Hazzard 2003). Recently, Stanfield and Hunter (unpublished data) have shown that in female black flies, honeydew sugars produce flights of longer distance and duration than do nectar sugars. This thesis examines two aspects of black fly biology as it relates to sugar meal consumption. First, the effects of honeydew and nectar on black fly longevity are examined. Second, the proximate causation behind longer flight performances in honeydew-fed flies will be examined.

The comparison between these two sources is important because nectar is composed of mainly simple sugars (monosaccharides and disaccharides) whereas honeydew is composed of both simple and complex sugars (including trisaccharides and tetrasaccharides).
Objectives

Black Fly Longevity

1. To test the hypothesis that the consumption of nectar sugars versus honeydew sugars differentially affects longevity in male and female black flies.

Proximate Causation Behind Longer Flight Performances in Honeydew-Fed Flies

2. To test the hypothesis that the consumption of simple sugars versus complex sugars differentially affects the flight performance of female black flies.

3. To test the hypothesis that the complex sugar melezitose is digested in female black flies and that melezitose is the causative agent behind the increased flight performances.
LITERATURE REVIEW

Black Flies

Black flies belong to the family Simuliidae and the order Diptera, commonly known as the true flies. Black flies first appeared in the upper Jurassic/lower Cretaceous periods and have existed on earth for approximately 160,000,000 years (Currie & Grimaldi 2000). Black flies can live virtually anywhere there is fresh running water, although they are most abundant in the north temperate and subarctic regions. Worldwide there are 1,800 known extant species of black flies and 25 genera, with approximately 254 of these species (13 genera) in North America (P.H. Adler, pers. comm.).

Overall, the family Simuliidae is a relatively small and homogeneous family (when compared to other dipteran families), making it an easily recognizable group (Peterson 1981). Black flies are probably most well known for their biting habits, as the females readily attack humans, birds, livestock, poultry and many wildlife mammal species. Black flies are responsible for the transmission of human onchocerciasis (river blindness), bovine onchocerciasis and avian leucocytozoonosis, making them serious vectors of disease. Onchocerca volvulus is a parasitic worm, that is transmitted to humans during black fly blood-feeding. A total of 18 million people are infected with Onchocerca volvulus, the causative agent responsible for causing river blindness (99 % of these people live in Africa). Of the 18 million people infected, 270,000 people are blinded by the disease (World Health Organization 2003).

Life Cycle
Egg Stage

Black flies exhibit complete metamorphosis and their life cycle consists of an egg, larva, pupa and adult stage (Figure 1). The immature stages (egg, larval and pupal stages) are aquatic and are most commonly found in rivers and streams (Burger 1987). Females lay their eggs on substrates (rocks, sticks and/or grass) present within the flowing water, or directly on the water’s surface. The egg stage is a stage of both development and rest. The duration of this stage is dependent on the environmental conditions and can be as short as one day or as long as ten months (as cited in Crosskey 1990). The environmental conditions that most affect the length of the egg stage are oxygen availability and water temperature. This stage is very important as many simuliids live in environments that have long periods of harsh and unfavourable conditions. Many species have evolved to use egg diapause as a period of rest to await more favourable environmental conditions.

Larval Stage

Black fly larvae are club-like and appear swollen at the posterior end. They are characterized by the presence of a pair of labral head fans and a single anterior proleg. Black fly larvae attach themselves to trailing grass or other substrates by spinning a pad of silk and then attaching its posterior circlet of hooks into it (Stuart & Hunter 1998). Black fly larvae require a constant flow of water, from which they are able to obtain oxygen and nutrients (Adler & McCreadie 1997). The oxygen diffuses through the insect cuticle of the larvae and the labral fans filter out the nutrients.
Figure 1 – A diagram showing the life cycle of black flies. The adult female black fly deposits her eggs on the trailing aquatic vegetation. The black fly larvae anchor themselves to the vegetation and are able to feed using their labral fans. The larvae then pupate and once conditions are favourable the adult fly emerges and floats to the top of the water surface (Wood 1985).
Larval growth is characterized by a series of moults that occur between successively larger instars. There are from six to nine instars, depending on the black fly species. The time required for the larval stage varies from 10 days to several months. Some black flies are able to overwinter in this stage (Davies et al. 1962).

**Pupal Stage**

Unlike the larval stage, black fly pupae are immobile and feeding does not occur during this stage (Magnarelli & Burger 1984). All black fly pupae have a pair of respiratory organs, called the gills, which enable gas exchange to occur during this stage. The pupal cocoon acts as a protective shield against desiccation when water levels fluctuate. The duration of this stage is thermally dependent and lasts approximately one week.

The energy required for emergence is generated from the nutrients acquired during the larval stage. Eclosion is initiated when the black fly begins to move about within the pupal cocoon. During this time the pupa fills with gas, causing pressure to build against the pupal cuticle. While still inside the pupal cocoon, the black fly expels air from its respiratory system, and breaks through the pupal cuticle. The black fly is then able to float to the water’s surface in an air bubble; it finds a suitable area along the water’s edge to allow its wings to dry (Davies et al. 1962).

**Adult Stage**

Adult black flies are small, darkly coloured flies with short legs, broad wings and a humpbacked appearance. Body lengths range from 1.2 mm to 6.0 mm. Most female mouthparts are of the biting and sucking type, whereas male black flies have reduced mouthparts. During adult life, male black flies are focused on finding a sugar meal and
mating. On the other hand, most female black flies must sugar feed, mate, blood feed and then find a suitable site for oviposition (blood feeding may then be repeated) (Adler & McCreadie 1997).

Habitat

The habitat of black flies varies with the stage of development as well as the species. The egg, larval and pupal stages are all aquatic, and are restricted to fresh flowing water, whereas the adult stage is typically terrestrial. The aquatic habitat is highly variable, ranging from small trickles to large flowing rivers. Black flies are highly adaptive organisms, allowing them to inhabit almost all types of fresh flowing water.

Generation Time

The duration of one generation of black fly (from egg to adult) varies with voltinism (number of generations produced annually), climate and geographic location. Although there are both univoltine species and multivoltine species, the majority of species in Canada are univoltine (Davies et al. 1962). Some of these species, primarily from the genus Prosimulium overwinter as larvae and are subsequently ready to pupate and emerge as adults in the early spring. Other univoltine species overwinter in the egg stage and emerge as adults in late spring or early summer. The length of time spent during the egg stage can vary from less than two days (warm tropical regions) to more than a year (long drought periods). Larvae persist for a minimum of four days to a maximum of several months (species that overwinter as larvae), and the length of the pupal stage ranges from two days to a maximum of two to three weeks (Crosskey 1990).
Few reliable studies have been conducted on the length of the adult stage in black flies in the wild. At most, female black flies can survive for two to three months (under optimum conditions) (Stanfield 2000); however, they most likely survive two to three weeks in the wild. Male black flies typically survive only a couple of days in the wild, although they are capable of surviving longer in captive longevity studies.

**Male and Female Black Flies**

Male and female black flies are easily distinguishable from one another. The most obvious difference between the two sexes is that the female head is dichoptic (eyes do not meet along the midline) whereas the male head is usually holoptic (eyes meet along the midline). The facets of the upper half of the male eye are distinctly larger than the facets of the lower half (Davies et al. 1962). As a result the male head appears spherical whereas the female head looks more hemispherical (Figure 2). Female mouthparts are adapted for cutting the skin and sucking blood, whereas male mouthparts (and the mouthparts of a few female species) are suitable only for the uptake of liquids, such as water and/or a sugar meal. Female tarsal claws (especially of ornithophilic black flies) are often specialized for blood-feeding purposes (allows them to manipulate the bird feathers), whereas male claws are more specialized for grasping female vestiture.

**Feeding**

Sugar is the basic food of adult black flies. Both sexes sugar feed, whereas only the female black fly blood-feeds (Anderson 1987; McIver & Sutcliffe 1987).
Figure 2 – A) A scanning electron microscope (SEM) of a typical blood-sucking female black fly (*Prosimulium susanae*) head and mouthparts. Photograph courtesy of D.A. Craig.

B) A photograph of a typical male black fly (*Simulium venustum*) head and mouthparts. The enlarged upper facets and much smaller lower facets of the eye are visible.
This is true for all haematophagous dipterans except the tsetse fly and stable fly where both the male and female flies blood feed. Sugar feeding provides the energy necessary for flight and increased longevity (Foster 1995). The protein gained from a blood meal aids in egg development (specifically vitellogenesis) in female black flies (Anderson 1987).

**Blood Feeding**

Although blood feeding among female simuliids could almost be considered universal, there are some exceptions. Blood is a source of protein, which triggers hormonal secretions required for egg maturation and provides materials for yolk synthesis (Wigglesworth 1949; Anderson 1987). A blood meal is physiologically essential for the development of eggs, except in the case of autogenous females. Autogenous female black flies are able to lay their first batch of eggs without blood feeding. The energy required for egg development is carried over from the immature stages. Autogenous black flies can be further divided into non-biting black flies (obligatory autogeny) and blood-sucking black flies (facultative autogeny). Worldwide there are approximately 37 species of black flies that are unable to blood-feed, because their mouthparts are reduced and not able to pierce the skin of a host (Davies et al. 1977). In the case of blood-sucking autogenous black flies (facultative autogeny), females do not require a blood meal for their first batch of eggs, but they do blood feed to mature any subsequent egg batches (Mokry 1980).

Blood-feeding habits and host preferences among simuliids are highly variable. Regardless of host type, blood feeding by simuliids generally occurs outside (exophilic)
and during daylight hours (diurnal). The frequency of blood feeding is dictated by the
gonotrophic cycle of the female black fly (Magnarelli & Burger 1984).

**Black Fly Blood-Host Relationships**

Two groupings are recognized when describing the relationship between black flies and their blood-meal host(s). Ornithophilic black flies feed on the blood of birds, and mammalophilic black flies feed on the blood of mammals. Some authors reserve the term anthropophilic for those mammalophilic black flies that feed on the blood of humans (Crosskey 1990). Many black flies are host specific, as the more specific the blood type the more specific the nutritional rewards will be (Downes 1958; Anderson 1987). There are some black flies, however, that are less specific than other black flies when it comes to acquiring a blood meal, so there is overlap among these three groups. Black flies that feed on both birds and mammals can be grouped together and called zoophilic black flies. Although the majority of black flies exhibit overlap among their host choice, there is one particular species of black fly, *Simulium euryadminiculum*, which feeds on the blood of the common loon or great northern diver (*Gavia immer*) (Fallis & Smith 1964).

**Host Location**

The bloodthirsty female black fly locates her host through both olfactory and visual cues (Sutcliffe 1986). Hosts are located by the scent trail (body odour and CO₂) that the black fly is able to detect. Black flies are probably able to locate their hosts from vision alone, i.e., seeing a host in an open field. Short-range host location can be attributed to sight, mainly host profile, colour and reflectance.
Sugar Feeding

The primary role of sugar feeding for black flies in nature is to increase longevity and energize flight (Hocking 1953; Davies et al. 1962; Dethier 1976; Crosskey 1990). Sugar sources that are available to black flies are floral nectar, sugars from extra-floral nectaries and homopteran honeydew. The carbohydrate component of each of these is thought to provide significant energy for flight and increases longevity.

Floral Nectar

Nectar is the sugary solution produced by the floral nectaries. The most common sugars found in floral nectar are fructose, glucose and sucrose (Figure 3) (Percival 1961; Baker & Baker 1983; Gottsberger et al. 1984; Stiles & Freeman 1993). Rarer sugars such as melibiose, maltose and raffinose are also found in small amounts (Baker & Baker 1983).

Sugars account for the bulk of the total dry weight of nectar, with the remainder being accounted for by phenols, amino acids, reducing acids, proteins, lipids, alkaloids and antibiotics (Baker & Baker 1983; Martini et al. 1990; Bernardello et al. 1994). Concentrated sugar can be obtained from the floral nectaries or from the extra-floral nectaries or non-floral parts of the plant. In general, small plants that are yellow and green in colouration with obscure flowers are most attractive to black flies (Wenk 1965; Hunter 1979). Black flies are thought to be opportunistic sugar feeders.

Homopteran Honeydew

Homopterans such as aphids (Aphidae), adelgids (Adelgidae), coccids (Kermidae), white flies (Aleyrodidae) and leaf hoppers (Cicadellidae) feed by piercing
Figure 3 – Structural diagrams of various carbohydrates (from Alberts et al. 1994; trehalose from Gilmour 1965).
plant tissue with their stylets and ingesting the sap (Bryne & Miller 1990). After feeding, these homopterans excrete honeydew, and this sugary waste product is left behind in abundance on the surfaces of leaves. Sugars make up more than 80% of the total dry weight of honeydew, with amino acids, amides, organic acids, alcohols, auxins and salts accounting for the remaining 20%. Honeydew composition can vary depending on the plant species and homopterans involved. Although many different sugars have been identified in honeydew, the most common are fructose, glucose, sucrose and melezitose (Volk et al. 1999; Wäckers 1999, 2001; Gamble 2002). Stachyose, raffinose, turanose, melebiose and maltose are also frequently encountered (Auclair 1963; Bryne & Miller 1990; Janzen & Hunter 1998; Wäckers 1999, 2001).

Especially in areas like the coniferous forests of Canada and Siberia where floral nectary abundance is low, homopteran honeydew probably plays a more significant role than floral nectar in black fly sugar feeding (Crosskey 1990).

**Carbohydrate Digestion**

When a sugar meal is ingested, it is intermittently released into the midgut from the crop (a sac-like storage organ). In contrast, when a blood meal is ingested, it moves directly to the midgut. Part of the sugar meal may be directed to the midgut if it is empty, but most of the sugar meal is directed to the crop. Glucosidases are the primary enzymes for digestion in insects that feed on plant-derived sugar sources (Dillon & Kordy 1997). The enzyme α-glucosidase acts on terminal α-linked glucose residues. The enzymes associated with carbohydrate digestion in sand flies (*Lutzomyia longipalpis*) that were fed a sugar only diet, a blood meal diet, and a mixture of the two diets, were investigated. In
total, seven different α-glucosidases were found (with isoelectric points ranging from 4.3-5.8), none of which were found in the crop, indicating that the glucosidases originated from the midgut epithelium rather than the salivary glands. Ninety percent of the enzyme activity was associated with the sugar-fed flies, as opposed to the 46% that were associated with the blood-fed flies. In another study, using mosquitoes (Aedes aegypti), in addition to the α-glucosidases found in the midgut, α-glucosidases were also found in the salivary gland (Marinotti & James 1990). However, another group of researchers followed up on this work and reported that although α-glucosidases were found in the salivary glands of Aedes aegypti, no enzyme activity was found in the mosquito saliva. These authors concluded that the α-glucosidase previously detected was for purposes of intracellular metabolism (Kerlin & Hughes 1992). Anez et al. (1994) showed, with the use of gas chromatography (GC) and high-performance liquid chromatography (HPLC), that when phlebotomine sandflies were fed disaccharides and trisaccharides, these sugars rapidly hydrolyzed into their constituent monosaccharides. Once the ingested sugar meal is broken down into monosaccharides (either fructose or glucose), these simple sugars are able to diffuse across the midgut wall. Once across the midgut wall these monosaccharides have various fates. They can be stored as glycogen, they can be degraded by glycolysis (which results in ATP or other precursors that can be used during lipid synthesis), or they can be converted to trehalose. One additional fate of dietary monosaccharides (although it has yet to be shown to occur in Diptera), is that they can be oxidized directly by the flight muscle and can form a major flight fuel. However, this work was done using the honeybee (Apis mellifera) (Woodring et al. 1993).
Flight

General Flight Conditions

Once a black fly has enough energy to fly (provided by sugar reserves or sugar feeding) the two main factors affecting flight are temperature and light intensity. Black flies fly mainly during daylight hours and when the temperature is 15 – 25 °C (Crosskey 1990). There is variation in the flight range of each individual black fly, which is highly dependent on sex and species.

During Flight

During flight, black flies cross their back two sets of legs over one another and fold their front set of legs close to their head. Black fly wings follow a figure eight pattern during flight (Figure 4). This pattern results in both pronation and supination, which allows for both wing surfaces to be exposed (Weis-Fogh 1973).

How diet affects flight

Williams (1943) has shown that a decrease in flight duration is due to a limitation in carbohydrates. Clegg and Evans (1961) have further shown that a flight (after exhaustion has been reached) is the result of the ingested carbohydrates or sugar meal, as opposed to any stored energy reserves. Trehalose is the main insect blood sugar, and is required by the flight muscles to fuel flight.

The Importance of Trehalose

Trehalose was first isolated from the fungus ergot and was known as a metabolite of fungi and many primitive plants (Gilmour 1965). Trehalose was rediscovered as a
Figure 4 – A diagram of *Simulium venustum* in full flight with the figure eight pattern that is created during flight indicated by a dotted line (Hocking 1953).
physiologically important sugar to insects in the mid-fifties (Wyatt & Kalf 1956, 1957). Trehalose is the major hemolymph blood sugar and, therefore, the main energy source of most insects (Gilmour 1965; Becker et al. 1996). It has a high concentration in the hemolymph, generally between 1% and 2% (0.06M) (Gilmour 1965; Becker et al. 1996; Candy et al. 1997). Trehalose is capable of existing in such high concentrations in insect hemolymph because it is a non-reducing sugar and is less reactive than other toxic reducing sugars, most notably, glucose (Becker et al. 1996; Candy et al. 1997).

**Synthesis of Trehalose**

Monosaccharides that are consumed in the diet or broken down from complex sugars are absorbed across the midgut epithelium by facilitated diffusion into the hemolymph. These monosaccharides (mainly glucose) are then removed by active transportation from the circulating hemolymph into the fat body (Candy et al. 1997; Stanley et al. 1997). These sugars are then assembled into glycogen and stored in the fat body (Becker et al. 1996; Stanley et al. 1997). The fat body is also the site of trehalose synthesis, the process by which the monosaccharides are converted to the non-reducing sugar, trehalose. The synthesis of trehalose traps the carbohydrate and promotes the uptake of other dietary monosaccharides from the gut (in the absence of an active transport system).

Candy et al. (1997) summarized trehalose synthesis in a concise, explicit diagram (Figure 5). The hydrolysis of individual glucose residues from glycogen, initiates trehalose synthesis. Phosphorylase yields molecules of glucose-1-phosphate, which are
Figure 5 – A diagram explaining trehalose synthesis and related reactions in the insect fat body. The enzyme pathways are also shown: (a) kinases; (b) glycogen phosphorylase; (c) phosphoglucomutase; (d) glucose 1-phosphate uridylyl transferase; (e) trehalose 6-phosphate synthase; (f) trehalose 6-phosphatase; (g) phosphoglucone isomerase; (h) phosphofructokinase-1; (i) fructose-1-6-bisphosphatase; (j) glycolysis; (k) gluconeogenesis. Figure taken from Candy et al. (1997).
then converted into glucose-6-phosphate and uridine diphosphoglucose (UDPglucose) (Candy et al. 1997; Stanley et al. 1997). These two glucose derivatives are then combined into trehalose-6-phosphate by trehalose-6-phosphate synthase. In a final phosphatase step, trehalose-6-phosphate yields a free trehalose molecule via trehalose-6-phosphatase (Candy et al. 1997; Stanley et al. 1997).
CHAPTER 1 – Longevity in Black Flies

Abstract

In the wild, black flies feed on both floral nectar and homopteran honeydew. I tested the hypothesis that the type of sugar diet ingested affects black fly longevity. All flies used in this experiment belonged to the *Simulium venustum* complex. All males used in the study were *Simulium truncatum* and 31% of the females were *Simulium truncatum* and 69% were *Simulium venustum*.

Newly emerged black flies were housed individually and were maintained on one of three possible diets: distilled water (dH2O), artificial nectar (AN) or artificial honeydew (AH). The life span (in days) was recorded for both male and female black flies and the sugar concentration for both the AN and the AH diets was 50% (w/v). The AN and AH recipes were designed to best represent natural nectar and natural honeydew.

Female *Simulium venustum* lived significantly longer on the AH diet than on the AN diet. Both females and males of *Simulium truncatum* maintained on the 50% sugar diet (AN or AH) lived significantly longer than flies maintained solely on dH2O. However, the type of sugar meal (AN versus AH) did not significantly affect longevity in either sex of this species.
<p>Text on the page.</p>
INTRODUCTION

The two main sources of sugar in the wild are floral nectar and homopteran honeydew. These two sugar sources differ in the types of sugars they contain. Floral nectar is composed of primarily simple sugars (monosaccharides and disaccharides), whereas homopteran honeydew contains complex sugars (trisaccharide and tetrasaccharides) in addition to simple sugars. The carbohydrate component of the sugar meal generally increases longevity (Davies et al. 1962; Hunter 1977; Magnarelli & Burger 1984; Sutcliffe 1986) and this is the reason why lab colonies of black flies (and other hematophagous Diptera) are fed sugar (Nayar & Sauerman 1971; Brenner & Cupp 1980; Bernardo et al. 1986; Gray & Noblet 1999). This is the first study to address the question of whether artificial nectar diets and artificial honeydew diets differentially affect longevity in male and female black flies.

Sugar Sources

Both floral nectar and homopteran honeydew are readily available food sources in the wild, but their sugar compositions vary from one another.

The specific composition of sugars in flower nectar is dependent on the plant species. Nectar is composed of simple sugars (monosaccharides and disaccharides), the most common of these being fructose, glucose and sucrose (Wykes 1952, 1953; Baker & Baker 1983; Freeman et al. 1991; Romeis & Wäckers 2000). Differences in the total concentration of sugars vary from nectar to nectar, with a relatively constant amount of fructose, glucose and sucrose found (Baskin & Bliss 1969). Sugar concentration of natural nectar varies with the plant species and the weather conditions (excessive rain
dilutes the concentration of sugar available in the nectar). Hocking (1968) compared the sugar concentrations of nectars produced by various plant species on Ellesmmere Island. The range of reported sugar concentrations of the floral nectar was as low as 5% and as high as 80% (w/v). In a study conducted by Schaefer & Miura (1972), the range of sugar concentration of floral nectar ranged from 20 to 50% (w/v). Galetto et al. (1998) investigated the sugar concentrations of floral nectar by analyzing 54 populations, which included 14 species and six varieties. The mean nectar sugar concentration of all samples was 48.4% ±19.8. (ranged from 12% to 84%).

Honeydew is composed of both simple (monosaccharides and disaccharides) and complex sugars (trisaccharides and/or tetrasaccharides). The most common sugars that are present in honeydew are fructose, glucose, sucrose and melezitose (Volkl et al. 1999; Wäckers 1999, 2001; Gamble 2002). Mittler (1958) also reported that honeydew is composed of roughly equal amounts of fructose, glucose, sucrose and melezitose. Yao and Akimoto (2001) determined that fructose, glucose, sucrose, trehalose and melezitose accounted for approximately 90% of the total volume of sugar in the homopteran honeydew. Although the types of sugars present in honeydew seem somewhat fixed, the sugar concentration of honeydew is quite variable; Grant and Beggs (1989) reported that the percentage of sugars in homopteran honeydew can vary from 5 % to 64% (w/v). The sugar concentration of honeydew varies depending on the host plant and the species of homopteran. The daily weather condition (i.e., amount of rainfall) is also a contributing factor to the sugar concentration of the homopteran honeydew.

**Black Fly Longevity Studies**
Diet Administration, Housing and Results

In a study conducted by Davies (1953), different methods of maintaining females of *S. venustum* in the lab were investigated. Higher survival was reported in female flies fed an *ad libitum* 10% (w/v) sugar solution, when compared to female flies fed a 0%, 50% and/or 100% (w/v) sugar solution. Davies (1953) also reported a higher survival rate among female flies that were provided with water and dry sugar crystals separately, compared with female flies fed the 10% (w/v) sugar solution. In these studies, black flies were housed either individually or in groups (up to 39 flies) in longevity tubes, which consisted of an 8 inch glass vial plugged with cotton (vials were modified slightly for each of the various feeding methods).

In a study conducted by Hazzard (2003), the longevity of female black flies was recorded. Flies were housed individually and provided with sugar solutions (either a 10% w/v nectar solution or a 20% w/v honeydew solution) *ad libitum*. She determined that sugar-diet type did not significantly affect longevity, although when the sugar diets were compared to the no-sugar diet, longevity was significantly affected.

Other Longevity Studies

Diet Administration, Housing and Results

Andersson (1992) conducted a mosquito longevity study, testing two different types of sugars (fructose and sucrose) at various concentrations to see if there were any differences in the average life span of the mosquito. For this study, female mosquitoes (*Aedes communis*) were housed individually and were supplied with one of the following diets: fructose or sucrose at 10%, 25% or 50%. The sugar diet was administered by
soaking cotton balls in the sugar solution. Mosquitoes lived significantly longer on the 25% and 50% sugar solutions of fructose and sucrose than on the 10% fructose and sucrose solutions. Although differences were seen between the different sugar concentrations of the diets, there was no significant difference between the two types of sugars.

In a mosquito longevity study conducted by Nayar and Sauerman (1971), groups of 300 female mosquitoes (Aedes taeniorhynchus) or 300 male mosquitoes were housed in cages (22 x 30 x 33 cm), and were provided with either a distilled water diet or a 10% w/v sugar solution. The diet was provided via a small vial covered with a piece of fine mesh cloth. The mean life span of the male and female mosquitoes on the two diets was then compared. Females lived longer than males (for both the distilled water diets and the 10% sucrose diets), and mosquitoes lived significantly longer on sucrose than on water alone (Nayar & Sauerman 1971).

Özalp and Emre (2001) looked at longevity of female Pimpla turionellae (Hymenoptera) after maintenance on various artificial diets. These insects were housed individually and were fed ad libitum. Each diet consisted of one type of carbohydrate (14%), and a mixture of amino acids, lipids, vitamins and inorganic salts (as described by Emre 1988). Among the diets tested there was no significant difference between the sucrose diet and the melezitose diet.

Black Flies

Prior to 1997, floral nectar was thought to be the main source of carbohydrates for black flies. However, Burgin & Hunter (1997a,b,c) demonstrated that up to 35% of
females of *S. venustum* had recently consumed meals of homopteran honeydew. This information has necessitated a re-assessment of many life history aspects, including longevity.

Because artificial honeydew meals contain complex sugars as well as the simple sugars that artificial nectar contains, I hypothesized that the complex sugars in honeydew would result in increased black fly longevity.

**Objectives**

This study was designed to test the hypothesis that the consumption of nectar sugars versus honeydew sugars differentially affects male and female longevity in black flies.

**METHODS**

**Diet Selection**

The artificial nectar and honeydew recipes (Appendix 1) were designed to best represent natural nectar and natural honeydew. Although the sugar concentrations of both floral nectar and homopteran honeydew are quite variable, the sugar concentrations chosen for the two artificial diets used in this study were 50% w/v, a value that lies within the ranges of reported sugar concentrations for both natural nectar and natural honeydew. By using the same sugar concentrations for both diets, I hoped to eliminate the possibility of seeing differences due to different sugar concentrations and expected to see only the differences due to the various sugars in the two artificial sugar diets.
Black Fly Collections

Research for this project was conducted at the Wildlife Research Station in Algonquin Provincial Park, Ontario (45°34’N, 78°41’W), from May 15th until July 20th 2001. Black fly pupae were collected from two sites: North Madawaska River at Sasajewun Dam and Mud Creek. Collections were carried out every other day, to ensure that there was a constant supply of newly emerging black flies. Pupae were collected from the streams, where they were most commonly attached to sticks, trailing grass or rocks. The substrates with the pupae attached were removed from the stream and were transported back to the lab and placed in rearing cages. The rearing enclosures were square wooden frame boxes (1 m by 1 m) covered in a fine cloth (e.g., Vista® curtain sheer material) (Hunter et al. 1994). One of the faces of the box had a sleeve, which allowed for access to, and removal of, the flies. To keep the pupae moist and to make sure that they did not desiccate, the pupae inside the rearing enclosures were spritzed with stream water three times daily.

Experimental Protocol

Female and male black flies were maintained on either dH2O or one of two 50% (w/v) sugar diets (AN or AH) (Appendix 1).

Holding Containers and Diet Administration

Newly emerged female and male black flies (no older than 12 hours) were individually placed in Monty vials (based on the rearing chambers in Hunter et al. 1994). Monty vials are modified 20-mL glass scintillation vials, that contain a Plastozote®
(styrofoam type material) bottom, a shell vial, a toothpick (providing a rest area) and a small glass coverslip (for diet administration) (Figure 1). Distilled water with a cotton plug was placed in the shell vial so that water was provided *ad libitum* throughout the study. Black flies were fed by placing 1 μl of diet (sugar or dH2o) on the glass coverslip every other day (done for the dH2o flies, so that they were all disturbed the same amount).

**Longevity**

Longevity, for the purpose of this study, is defined as the number of days that an adult black fly survived. Flies were checked every 12 hours and any deaths were recorded. At the end of this study, the total number of days that the black flies survived on each of the three diets was obtained. If a fly died from what appeared to be unnatural causes, such as getting stuck in the diet or caught underneath the coverslip, it was discarded and a new fly was started in another vial.

**Species Identifications and Wing Measurements**

All black flies used in this study were identified to species, using a dissecting microscope and the identification key of Davies *et al.*(1962) and Hunter (1990). Wing
Figure 1 – A photograph of a female *Simulium venustum* black fly in a Monty vial during the longevity study. The shell vial with a cotton plug contained distilled water, the toothpick provided a rest area for the black fly and the small circular coverslip provided an area to administer the 1 μL of diet.
measurements of each fly were also taken by removing the right wing of each fly and measuring (using an eyepiece micrometer) the distance from the humeral crossvein to the apex of the wing (Figure 2).

**Statistical Analysis**

The average longevity values of flies maintained on the three diets were compared using a One-Way ANOVA with a post hoc Tukey HSD comparison of means test.

**RESULTS**

All flies used in the longevity study belonged to the *Simulium venustum* complex flies (100% of males were *S. truncatum*, 31% of females were *S. truncatum* and 69% were *S. venustum*). It was determined post hoc that flies on the different diets in each of the experiments did not differ in wing length: female *S. venustum* (*F*<sub>2,46</sub>=0.14, *p*=0.8698), female *S. truncatum* (*F*<sub>2,19</sub>=0.45, *p*=0.6465), and male *S. truncatum* (*F*<sub>2,45</sub>=0.53, *p*=0.5902) (Table 1).

**Average Longevity for Female Black Flies**

The average longevity of 71 female black flies fed three experimental diets was recorded; of these, 69% were *S. venustum* and 31% were *S. truncatum*. Female *S. venustum* fed the dH<sub>2</sub>O water diet survived 2.96 ± 0.36 days, with no flies surviving longer than four days (Figure 3). Females of *S. venustum* maintained on the 50% w/v AN diet survived 10.69 ± 6.30 days, with no flies surviving longer than 24 days. Females of *S. venustum* maintained on the 50% w/v AH diet survived 20.00 ± 12.23 days, with no flies surviving longer than 36 days. There was a significant difference
Figure 2 – Wing measurements demonstrated on a male *Prosimulium fuscum* wing.
Table 1 – Wing measurement values (measured in mm, given as mean ± SE) for all species of male and female black flies on each of the three diet types (dH20, AN and AH).

<table>
<thead>
<tr>
<th>Species (N)</th>
<th>Distilled Water (dH20)</th>
<th>Artificial Nectar (AN)</th>
<th>Artificial Honeydew (AH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>female <em>S. venustum</em> (49)</td>
<td>2.57 ± 0.03</td>
<td>2.59 ± 0.03</td>
<td>2.58 ± 0.04</td>
</tr>
<tr>
<td>female <em>S. truncatum</em> (22)</td>
<td>2.60 ± 0.04</td>
<td>2.54 ± 0.05</td>
<td>2.69 ± 0.05</td>
</tr>
<tr>
<td>male <em>S. truncatum</em> (48)</td>
<td>2.37 ± 0.02</td>
<td>2.34 ± 0.02</td>
<td>2.37 ± 0.04</td>
</tr>
</tbody>
</table>
Figure 3 – Average longevity values (measured in days ± SE) for female *S. venustum* black flies fed one of three experimental diet types: dH2O, 50% w/v AN or 50% w/v AH.
between all three diets types (F2,46= 25.60, p<0.0001). Flies survived significantly longer on the AH diet when compared with the AN and dH20 diets. The AN fed flies also survived significantly longer than the flies fed dH20.

On average, females of *S. truncatum* fed the dH20 water diet survived 2.67 ± 0.52 days, with no flies surviving longer than three days (Figure 4). Female flies maintained on the 50% w/v AN diet survived 18.64 ± 7.68 days, with no flies surviving longer than 34 days. Female black flies maintained on the 50% w/v AH diet survived 15.60 ± 11.39 days, with no flies surviving longer than 31 days. There was a significant difference between the sugar versus no sugar diets (F2,19=8.69, p=0.0021). Females of *S. truncatum* survived significantly longer on the AN and AH diets when compared with the dH20 diet.

**Average Longevity for Male Black Flies**

The average longevity of 48 male black flies (all were *S. truncatum*) fed the three experimental diets was recorded (Figure 4). On average, male black flies fed the dH20 water diet survived 2.70 ± 0.56 days, with no flies surviving longer than four days. Male flies maintained on the 50% AN diet survived 11.94 ± 7.12 days, with no flies surviving longer than 28 days. Male black flies maintained on the 50% AH diet survived 12.33 ± 6.02 days, with no flies surviving longer than 23 days. There was a significant difference between the sugar versus no sugar diets (F2,45=22.46, p<0.0001). Male black flies survived significantly longer on the AN and AH diets when compared with the dH20 diet.
Figure 4 – Average longevity values (measured in days ± SE) for female and male *S. truncatum* black flies fed one of three experimental diet types: dH20, 50% w/v AN or 50% w/v AH.
Effects of Diet on Longevity

Longevity was significantly influenced by diet type. A post hoc Tukey (HSD) comparisons of means test showed that there were three significantly different groups, for females of *S. venustum* (F$_{2,46}$=25.60, p<0.0001). Flies fed the AH diet lived significantly longer than flies fed the AN and dH$_2$O diet, and flies fed the AN diet lived significantly longer that the flies that were maintained on the dH$_2$O diet. Both females (F$_{2,19}$=8.69, p<0.0001) and males (F$_{2,45}$=22.46, p<0.0001) of *S. truncatum* fed the AN and AH diets lived significantly longer than the black flies that were fed the dH$_2$O diet.

DISCUSSION

Longevity Comparisons

Artificial nectar and honeydew diets significantly affect longevity in females of *S. venustum*. The flies that were maintained on the artificial honeydew diet survived almost twice as long as the flies that were maintained on the artificial nectar diet. Although significant differences between the two sugar diets were seen in the females of *S. venustum*, no significant differences were observed between the two sugars diets in either sex of *S. truncatum*.

Effects of Diet Administration

In my study the amount of diet provided to the flies was minimized, as opposed to other studies where diets were provided ad libitum. Ad libitum treatments might mask potential differences between diets because unlimited amounts of diet (regardless of the quality of diet) provide adequate amounts of energy to sustain longevity. However, if
there were differences between two diets, such as honeydew and nectar, they would be more likely to be visible under conditions of limited diet.

In a study by Woodhouse and Hunter (unpublished data), male and female black flies were maintained on either natural blueberry nectar (ad libitum) or natural adelgid honeydew (ad libitum) and their mean longevity (measured in hours) was recorded. They found that both males and females of *S. venustum* survived longer on honeydew than on the distilled water diet; however there was no significant difference between the flies fed the nectar and the distilled water diet. These same results were also observed in females of *S. truncatum*. Although significant differences were not seen between the nectar and honeydew diets, this study suggests that honeydew is better than nectar (relative to distilled water).

**Effects of Larval Reserves on Longevity**

The differences seen between the two species of black flies may be explained by the differences in the amount of reserves that are obtained in the larval stage, which might relate to their larval positioning within streams. In general, *S. truncatum* larvae favour outflows of streams (0-200 m below an outflow) (McCreadie & Colbo 1993). The outflow areas are generally nutrient rich and preimaginal development occurs most rapidly at this location (when compared to downstream sites along the same river) (Hunter 1990). *S. venustum* larvae on the other hand are referred to as ubiquitous in their distribution along a stream, i.e., they can be found at both outflow and downstream sites alike (Hunter 1990). As *S. truncatum* larvae have a preference for placement at outflows, there is likely competition at this location. Therefore, most *S. venustum* flies are not
located at the outflow centre. As a result, when flies are well fed in the larval stage (located at the outflow region of a stream), they emerge with adequate reserves and differences between two types of sugar diet are not observed. However, when flies are not as well fed in the larval stage (located downstream), they emerge with fewer reserves and are at a more critical stage of requiring a sugar meal, and differences between the two types of sugar diet are observed. These differences however, will vary between species and even within a species, depending on the larval location within the stream (as it relates to food availability).

Conclusions

This research project focused on studying whether or not nectar sugars versus honeydew sugars differentially affect longevity in males and females of the *S. venustum* complex. When only the carbohydrate components of these two diets are used, there is a difference in the longevity of females of *S. venustum*, but not in males or females of *S. truncatum*.

Longevity might not be solely dependent on the carbohydrate component of the sugar meals that black flies obtain. Although carbohydrate is necessary, it is not the only factor that is affecting longevity. The effects of carbohydrates on longevity also might depend on the species of black fly involved and the amount of reserves obtained during the larval stage. From the present study, the relationship between diet and longevity is still not complete and it is possible that components other than carbohydrates alone possibly play a role in determining longevity.
**Future Research**

In future studies it would be informative to look at the effects of nectar and honeydew, using parous flies of various species with low larval reserves, as it is suspected that differences between these two diets will be visible under these conditions. The comparison between the effects of nectar and honeydew meals (including all components of these two diets such as lipids and amino acids) on black fly longevity would also be of interest to investigate in future research projects.
CHAPTER 2 – Flight and Digestion of Various Sugars in Female Black Flies

Abstract

In the wild, black flies (Diptera: Simuliidae) feed on both floral nectar and homopteran honeydew, and in the lab artificial honeydew meals have been shown to produce significantly longer flights than artificial nectar meals. No previous study has investigated why honeydew sugars produce significantly longer flight distances and durations. All flies used in this study belonged to the Simulium vittatum Zetterstedt cytospecies IS-7.

The flight performances of exhausted individual black flies fed various sugars were recorded, using a computerized flight mill. The sugars tested were fructose, melezitose and combinations of fructose and melezitose. In addition, the digestion of these same sugars and other sugars was tracked, using thin layer chromatography (TLC).

Following a dH2O meal, exhausted flies were not able to regain flight. However, exhausted flies regained flight after a sugar meal with the exception of the melezitose-only meal. Flies flew farther on the fructose/melezitose mixture than on the fructose-only diet.

The TLC experiment showed that melezitose was broken down in flies fed the fructose and melezitose mixture diet. However, digestion was not initiated in the melezitose-only flies until 1.5 hours post ingestion. Together, the flight and TLC experiments suggest the breakdown of melezitose is aided by the presence of a simple sugar, namely fructose, and that the breakdown itself takes a long time. I conclude that it is the complex sugar, melezitose, accounts for the differences seen in the flight performance studies.
INTRODUCTION

Female black flies are well known for their blood-feeding habits. The protein that is acquired during a blood-meal is needed by female black flies to mature their developing eggs. Sugar is an additional food source that is required by both male and female black flies. The energy gained from these carbohydrate meals fuel flight and increase longevity. Black flies sugar feed from both floral nectar and homopteran honeydew (Burgin & Hunter 1997a,b,c). These two sugar sources vary in their carbohydrate compositions; floral nectar is composed of mainly simple sugars (monosaccharides and disaccharides), whereas homopteran honeydew is composed of complex sugars (such as trisaccharides and tetrasaccharides) in addition to the simple sugars. In a previous study, Stanfield (2000) showed that artificial nectar sugars and artificial honeydew sugars differentially affect flight performance in female black flies. This is the first study to address the question of what is causing these differences. It is hypothesized that the complex sugars in the honeydew cause the extended flight distances and durations.

Importance of Sugar Feeding

Although the specific habits of sugar feeding may vary from species to species, sugar feeding occurs frequently and the energy gained is used to fuel flight. Different types of sugar diets should be able to differentially affect flight performance, depending on the types and amounts of sugars ingested. The most common sugars found in floral nectar are fructose, glucose and sucrose (Wykes 1952, 1953; Baker & Baker 1983; Freeman et al.1991; Romeis & Wäckers 2000). The sugars most often encountered in
honeydew are similar to the sugars present in nectar, with the addition of melezitose (Volkl et al. 1999; Wäckers 1999, 2001; Gamble 2002). Melezitose is a trisaccharide and should be able to provide additional energy for flight. Sugar concentration of both nectar and honeydew is variable depending on the species involved (plant and homopteran); however, an average sugar concentration for both sources is approximately 50% (w/v) (Hocking 1968; Schaefe & Muira 1972; Grant & Beggs 1989; Galetto 1998).

**Flight Mill Systems**

Laboratory flight studies can serve as reliable indices of the flight potential of many insects. Flight mill studies were first used to answer questions surrounding mosquitoes of medical importance, mainly the anopheline mosquitoes that transmit malaria. They have helped in the mosquito control and abatement programs. Laboratory flight mill studies are beneficial, because field observations of flight are very difficult, if not impossible. Flight studies have many important implications such as testing the flight range of an insect that is a vector of disease agents, or testing the migratory abilities of locusts, to determine how long they are capable of travelling. Not only are the dietary factors that affect flight being studied but also the environmental factors.

Most flight mill systems that are used today are based on the flight mill system that is described by Rowley et al. (1968). Although individual systems may vary from one another, Hocking (1953) lists some requisites for an insect flight mill system: (1) low aerodynamic and frictional drag, (2) convenient and readily adjustable attachment procedure, (3) durability and (4) low moment inertia. One additionally important feature that is needed in a flight mill system is an accurate and reliable method of data recording.
Most flight mill systems that are used today are based on the descriptions of the flight mill system by Rowley et al. (1968) and Clarke et al. (1984). The paper by Clarke et al. (1984) provided descriptions about a microcomputer that provides an improved method of data recording. Like this method most flight mill systems today include a computer.

**Flight-Mill Studies**

Many flight mill studies have used mosquitoes and investigated the effects of various factors, such as the presence of a blood meal, parasitic infections and the reproductive stage on flight potential. In a flight mill study (based on the system described by Rowley et al. 1968) by Nayar and Sauerman (1973), mosquitoes (Aedes taeniorhynchus, Mansonia titillans, Culex nigripalpus, Psorophora confinnis and Aedes solitans) maintained on a 10% sucrose solution (ad libitum) reached their maximum flight potentials (longest flight distances in a 4.5 hour time interval) 2-8 weeks after eclosion. These researchers also looked at non-flown female mosquitoes (fed the 10% sucrose solution ad libitum) and determined the amount of glycogen that was present over the lifespan of these mosquitoes. They found that high levels of glycogen were reached during the second week after eclosion and these levels remained relatively stable for the duration of the mosquitoes’ lives. Nayar and Sauerman (1971) concluded that the elevated glycogen levels are responsible for the increased flight potentials seen in these older mosquitoes.

In another flight mill study (based on the Rowley et al. 1968 system), the flight capabilities of Aedes sierrensis mosquitoes were investigated. In total, there were three different comparison groups; mosquitoes that were infected with a parasite (Lambornella
clarki), females that contained a blood meal, and gravid mosquitoes. The flight capabilities of these three groups of mosquitoes did not significantly differ from one another, and the researchers concluded that the parasite in the mosquitoes did not affect the resources needed for flight (Yee & Anderson 1995). Briegel et al. (2001) supported the work by Yee and Anderson (1995) by showing that female Aedes vexans mosquitoes reached their flight potentials (longest flight distances flown in one night) of 10-17 km two weeks post eclosion and that this paralleled their peak in stored reserves (mainly glycogen).

Black Fly Flight Mill Studies

Hocking (1953) fed female Simulium venustum and Simulium vittatum a 25% glucose solution and recorded flight speed. He reported average flight speeds of 1.52 m/s for Simulium venustum flies and 2.47 m/s for Simulium vittatum flies. In a study by Cooter (1982), Simulium ornatum was fed a 10% sucrose solution and the average reported flight speeds were 0.38 m/s. In a fourth year thesis project by Gervan (1997) the effects of various sugars on flight performance in Simulium rostratum were investigated. When flies were fed 1 μL of diet, flies flew significantly faster on fructose and glucose than on sucrose. The average flight speed for Simulium rostratum was 0.28 m/s.

Sugar-Meal Digestion

During dipteran sugar-meal digestion, dietary carbohydrates are hydrolyzed in the gut and are broken down primarily into monosaccharides. These monosaccharides are then removed from the insect haemolymph by the fat body (Clegg & Evans 1961).
fat body is the only tissue that is able to use these sugars as well as endogenous reserves to synthesize hemolymph trehalose, a disaccharide composed of two glucose molecules. Once the trehalose reaches the flight muscles, it is broken down into glucose, which is then used to fuel flight, producing carbon dioxide and water as metabolic waste by-products (Clegg & Evans 1961).

The time required to resume flight for an exhausted fly depends on the rate at which the sugar meal is converted into blood trehalose. Wigglesworth (1949) found that many sugars have the ability to restore continuous flight after ingestion. The sugars that he tested were glucose, sucrose, trehalose and fructose. Glucose was capable of restoring continuous flight within 30-45 sec after ingestion, whereas sucrose and trehalose were capable of restoring continuous flight after 1 – 1 ½ min. The least efficient sugar was fructose, which restored flight 2 – 3 min after ingestion.

Sugar Detection Methods

Both high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) are methods used for separating and identifying different sugars. The HPLC technique is an elaborate technique and the equipment required to run an HPLC machine is expensive. HPLC machines are also connected to a computer, so that the samples are analyzed by the computer directly. Training is required for using an HPLC machine and the computer system used to run the setup. The HPLC technique is very sensitive in separating oligosaccharides. However, one drawback of HPLC is that it is not as sensitive at identifying monosaccharides (fructose and glucose) and it has a
tendency to overlay these simple sugars (Moore et al. 1987). For HPLC to run as effectively as possible, sample volume must be small (nL).

The TLC technique is a simple and cheap method that also allows the experimenter to determine the different sugars in a mixed sample. TLC is effective at accurately identifying both monosaccharides and oligosaccharides. This is achieved by comparing both a colour reaction (when the D.A.P.A reagent is used) and the migration distances of the sugar spots.

Although HPLC is considered to be a more sensitive technique than TLC, I chose to use the TLC. This method provided a very efficient method in terms of the information acquired, cost and time for studying the sugars from a large number of individual flies. Although this technique is not as sensitive as HPLC, the samples that I used contained debris (insect parts), and for the purposes of this study the abilities of TLC were not limiting. TLC is the technique that is widely used within our lab (Burgin 1996, Russell 2000). Also, the volume of the samples needed to be analyzed are in µL, which is a volume that is too large to run through the HPLC machine (samples were not concentrated enough to take a smaller amount).

**Black Flies**

Stanfield (2000) showed that honeydew-fed flies were able to fly significantly longer in distance (five times longer) and duration (four times longer) than nectar-fed flies. The cause of these differences has not been further investigated.
Objectives

This study was designed to test the hypothesis that the consumption of a simple sugar (fructose) versus a complex sugar (melezitose) differentially affects the flight performance of female black flies. A digestion experiment was incorporated to aid in the explanation of the differences in the flight performance experiment.

METHODS

Black Fly Collections

The black flies used in this study were from a colony, at the University of Georgia (Athens, Georgia). The black flies in this colony are Simulium vittatum Zetterstedt cytosome IS-7 (Brockhouse & Adler 2002). This colony of Simulium vittatum originated from eggs taken from a small stream in Cambridge, NY, at the New York State Experiment Station in September 1981. The original colony was located at Cornell University and was maintained by M.J. Bernardo, E.W. Cupp and F. Ramberg. In 1988 the colony was moved to the University of Arizona. Colony eggs were provided to Clemson University in March 1991. Under the supervision of R. Noblet and E.W. Gray the colony has been in continuous maintenance since 1991; in July 1999 the colony was moved to the University of Georgia (E.W. Gray, personal communication) (Figure 1). The Georgia colony is now the only laboratory colony of black flies in North America.

Each day newly emerged black flies were removed from the emergence tube (Figure 2) and placed into a – 20°C freezer for 1.5 minutes. After this time the black flies were sorted by sex and the females were individually placed into 1.5-mL Eppendorf
Figure 1. A photograph showing the University of Georgia aquatic runway used to maintain the IS-7 colony: a, fine pieces of fine mesh cloth with the black fly eggs are pinned to the runway; b, small larvae attached to the plastic lip of the runway; c, masses of black fly larvae attached to the runway.
Figure 2. A cage with an emergence tube (shown in the upper right of the photograph) attached. The cage is necessary to cover the aquatic runway once the black fly larvae are ready to pupate. The newly emerged adult black flies are attracted to the light and crawl/fly into the emergence tube.
tubules, and the males were discarded. The female flies were then used for either the flight mill or digestion experiment.

Sugar Diet

The two sugars chosen for the flight mill and digestion experiments were fructose (a representative simple sugar) and melezitose (a representative complex sugar). Fructose is commonly found in both natural nectar and natural honeydew, and melezitose is commonly found in honeydew. Various mixtures of these two sugars were tested in addition to the individual sugars.

Wing Measurements

The length of the right wing was measured (from the humeral crossvein to the apex of the M₁ vein) under a dissecting microscope equipped with an eyepiece micrometer (Figure 2 of Chapter 1). Wing measurements were made to ensure post hoc that flies had been placed at random into each of the experimental groups. To compare individual size variation, wing length is the best measurement (Crosskey 1990). Wing length remains constant and independent of the physiological state of the fly, whereas body length can be affected by the presence of eggs or a blood meal.

Statistical Analysis

Flight distance, speed and duration were analyzed using a One-Way ANOVA with a post hoc Tukey (HSD) comparison of means test.
Part One: Flight Mill Experiment

Flight Mill

A computerized flight mill (Figure 3) was used to record flight distance, speed and duration. The flight mill consisted of a retort stand, in which the supporting rod was 24 cm long by 1 cm in diameter. Mounted on the top of the rod was a sapphire frictionless pivot, which supported a 20-cm long rotating tether arm. A counter weight was added to balance the tether arm. Four reflective strips (placed 90° apart) were attached at the top of the pivot on the tether arm. A photoreceptor was mounted at the top of the stand, which allowed recordings to be taken every quarter rotation as one of the four reflective strips passed overhead. The photoreceptor was attached to an IBM PC equipped with a custom-designed DOS data acquisition/timer board and software (Brock University Electronics Shop).

During the flight, the computer created a spreadsheet that recorded flight distance (cm) and flight speed (cm/s) every quarter rotation. At the end of the flight, the data file was converted into a Microsoft® Excel (1997 version 5.1) spreadsheet. Once all data were in an Excel spreadsheet, areas of no recordings (when the fly was not flying) were removed. The majority of these areas of no recording occurred at the beginning of the flight when the fly had to be manually stimulated to begin or re-start flight, or at the end of the flight when exhaustion was nearing. Once all the zeros were removed, the total flight distance, speed and duration values were calculated.
Figure 3. The computerized flight mill consisting of: a, pinwheel design bottom; b, photoreceptor; c, computer; d, counter weight; e, reflective tape; f, flight mill arm; g, triangular cue card; h, close up photograph of triangular cue card glued to the thorax of a female black fly. The black fly is flying in this picture however the wings are beating so fast that they are not visible at this shutter speed.
Black Fly Preparations

Individual female black flies were placed in their 1.5-mL Eppendorf tubules in a –20°C freezer for 1.5 minutes. Once the fly was immobilized, the pointed end of an isosceles triangular shaped piece of cue card (0.75 cm at the base and 3 cm in height) was glued to the thorax of the black fly, using multipurpose non-toxic white glue (Figure 3, h). The tethered fly was then attached to the flight mill arm by the broad end of the cue card. Each fly that was used in this study was flown twice, first in an “exhaustion flight” and then in a “sugar flight”.

Exhaustion Flight

Black flies that were used in this study were randomly and blindly assigned to a sugar treatment prior to the exhaustion flight. The initial flight for each of the flies was termed the exhaustion flight. Tarsal stimulations consisted of manually stimulating the fly’s tarsi, and they were often required for the fly to regain flight. Exhausted flies commonly arrested flight with their wings extended in the flight position. Wigglesworth (1949) noted that as flies approached exhaustion they began to rest with increasing frequency until they had to be re-stimulated every minute or so. The exhaustion flight was used to deplete the fly of any stored glycogen reserves, thus ensuring that all flies began the sugar flight at the same physiological state. Exhaustion was reached once three tarsal stimulations would not result in flight.

Sugar Flight

Once flies had been exhausted, they were fed 1.0 μl of one of eight possible
null
diets. The sugar diets that were used consisted of fructose, melezitose or various mixtures of the two sugars. The stock solutions of fructose and melezitose were made by placing 50 g of sugar (either fructose or melezitose) into 100 mL of distilled water. The mixture diets were made by combining various amounts of the two 50% w/v stock solutions. For example, the 95partsfructose/5partsmelezitose (95/5) diet was made by adding 95 mL of the 50% w/v fructose stock solution to 5 mL of the 50% w/v melezitose stock solution. It was from this solution that the 1 µL that was fed to each of the flies was taken. Diets were made and diets are always reported with the fructose component first. The flies that were fed the fructose-only diet and melezitose-only diet were fed 1 µL from the 50% w/v stock solutions. The eight diets were as follows: distilled water (dH2O), fructose (fru), 95partsfructose/5partsmelezitose (95/5), 50partsfructose/50partsmelezitose (50/50), 40partsfructose/60partsmelezitose (40/60), 25partsfructose/75partsmelezitose (25/75), 5partsfructose/95partsmelezitose (5/95), or melezitose (mez).

Two additional diets were tested (the sample sizes of these two diets were very small and were omitted from statistical analysis). The first one (made in the same way as the above mentioned diets) was 75partsfructose/25partsmelezitose (75/25). The other diet was a 25% w/v sugar solution and the stock solution of this diet was made by mixing 25 mL of the fructose stock solution with 75 mL of distilled water (25fru).

The use of the dH2O diet acted as a negative control to ensure that the flight performances that resulted after the fly was fed a sugar meal were the result of the ingested sugar and not from any residual glycogen reserves. After the fly had been fed one of the diets, a 15-minute rest period was given, to allow adequate meal digestion. During the resting period, the fly was not permitted to fly; this was done by placing the
fly's tarsi on a flat surface. If a black fly's tarsi are touching something, it will not fly; however, once they are not touching anything (i.e., suspended in the air) the fly will usually begin flying (although sometimes tarsal stimulations are required). Once the resting period was complete, the sugar flight was initiated; again tarsal stimulation was required. During the sugar flight, flight distance, speed and duration were recorded on the computer.

**Part Two: Sugar Digestion Experiment**

The digestion of eleven different sugar diets was recorded over time (0, 1, 2, 5, 10, 15, 30 and 60 min). In total, 417 flies were used, with approximately 40 flies on each diet (5 flies at each different time interval).

**Black Fly Preparations**

Individual female black flies were tethered, as in the flight experiment, to facilitate handling of the flies.

**Sugar Meal Diets**

The diets were made in the same manner as described in the flight performance experiment (sugar concentration for all diets was 50 % w/v). Each fly used in the experiment was fed 1 µl of one of the eleven diets: distilled water (dH2O), fructose (fru), glucose (glu), sucrose (suc), 95partsfructose/5partsmelezitose (95/5), 50partsfructose/50partsmelezitose (50/50), 40partsfructose/60partsmelezitose (40/60),
25parts fructose/75parts melezitose (25/75), 5parts fructose/95parts melezitose (5/95), melezitose (mez) and stachyose (sta).

Diet Administration and Timed Intervals

Each fly was fed 1 μL of diet and once the entire diet was consumed the timed interval began. Flies were then sacrificed at the end of the timed interval by placing them in the −20 °C freezer at 0 min (fed no sugar), 1, 2, 5, 10, 15, 30 or 60 minutes post ingestion. In total, 5 flies for each time interval on each of the diets were used. To ensure that the interval of time post ingestion was as accurate as possible, flies were fed individually for the 1, 2 and 5 minute intervals, and in groups of 2 for the 10 and 15 minute intervals and in groups of three for the 30 and 60 minute intervals. For the melezitose-only fed flies, an additional time period of 1.5 hours, 2 hours and 3 hours was added. The sugar or sugars present in the flies at the time of death were then analyzed using TLC analysis at Brock University.

Black Fly Dissection

Black flies were dissected on microscope slides under a dissecting microscope. Micro-dissection scissors were used to cut through the female black flies at two spots; the first was just anterior to the hind legs, and the second was between abdominal segments seven and eight. The head, wings and legs were also removed using the micro-dissection scissors. After dissection was complete, each fly carcass was placed into a numbered well of a tissue-culture plate. Once an adequate number of flies (20 flies) was dissected to run
a TLC plate, 5 µL of distilled water was added to each well and the mixture (the insides of the black fly carcass and distilled water) was macerated.

**Thin Layer Chromatography (T.L.C)**

**Plates**

TLC plates were prepared using Sigmacell® type 20 (cellulose powder), according to the manufacturer’s instructions. A plate spreader was used to apply a 0.5-mm layer on the 20 cm by 20 cm glass plates.

**Standards & Black Fly Samples**

In addition to the black fly samples that were spotted along the bottom of the TLC plate, standard sugar solutions were also used. The use of the standards allowed for accurate identification of the sugars present within the black fly samples. The standards were applied at three different areas on the plate (both ends and in the centre), which helped to eliminate error due to edge effects. Both standards and samples were applied (1.5 µL) to the plate by using a Disposable Drummond Microcap® with a wire plunger.

The standard sugar solutions used are the ones commonly used in our lab. Both solutions were made by placing 0.1 g of each sugar into 10 mL of distilled water.

- Standard # 1 contained fructose, glucose, sucrose, maltose, maltotriose and melebiose.
- Standard # 2 contained turanose, melezitose and stachyose (Appendix 2).

**Solvent and Reagent**

The solvent used in this experiment consisted of 15 mL of formic acid, 25 mL of methyl ethyl ketone, 35 mL of tertiary butanol and 25 mL of distilled water (Damonte et al. 1971). The solvent (100 mL) was poured into the developing chamber prior to placing
the TLC plate inside for approximately two hours, or when the solvent front was approximately 2 cm from the top of the plate.

After each plate was removed from the developing chamber, it was placed into the fume hood where it was allowed to air-dry. The plate was then sprayed with the D.A.P.A. reagent (until the plate was fully saturated in the reagent but before sections of the cellulose began to peel off), using a Crown® spray nozzle attached to a 250-mL Erlenmeyer flask. The D.A.P.A. reagent contained 2 g of diphenylamine, 2 mL of aniline, 15 mL of phosphoric acid and 100 mL of acetone (Damonte et al. 1971). The plate was allowed to air-dry for 5 minutes and then it was heated with a flameless heat gun until the sugar spots appeared (approximately 5 minutes). This reagent reacted with the sugars and provided different coloured spots and hRf values (migration distance from the point of origin to the centre of the sugar spot, divided by the migration distance from the origin to the solvent front, all multiplied by 100), for each for each of the sugars. The colouration of the spots, hRf values and the comparison to the known sugars on the standard sugar solutions aided in the identification of each of the sugars. All sugars in the standards (except trehalose) undergo a colour reaction with the D.A.P.A. reagent.

RESULTS

Part One: Flight Mill Experiment

Flight distance, speed and duration were recorded for a total of 94 females of Simulium vittatum. Approximately 11 flies were flown on each of the eight experimental diets (dH20, fru, 95/5, 50/50, 40/60, 25/75, 5/95 and mez). Two additional diets were tested; 75/25 (75partsfructose/25partsmelezitose - sugar concentration was 50% w/v) and
the 25fru diet (sugar concentration was 25% w/v). These two diets were omitted from statistical analysis because their sample sizes were too small (n = 4, for both). Because none of the dH20 flies were able to regain flight after the exhaustion flight, they were also omitted from subsequent statistical analyses. Flies were placed at random on each of the ten diet treatments, with respect to wing length (F9,83=0.61, P = 0.7874) (Table 1).

Effects of Diet on Flight Distance, Speed and Duration

Diet significantly affected flight distance in female black flies (F6,92=20.52, P < 0.01). Flies were able to fly an average of 1919.8 ± 957.96 m on the fru diet and 1858.7 ± 1018 m on the 95/5 diet. Flies fed the 50/50 diet flew an average of 3529.0 ± 1913.6 m, whereas the flies fed the 40/60 diet flew an average of 1578.5 ± 560.63 m. Flies were unable to regain flight when fed the mez and 5/95 diets. Flies flew an average flight distance of 620.84 ± 343.88 m on the 25/75 diet (Figure 4).

Diet significantly affected flight duration in female black flies (F6,92=16.16, P < 0.01). Flies were able to fly an average of 5004.3 ± 2827.5 s on the fru diet and 5528.4 ± 5910.4 s on the 95/5 diet. Flies fed the 50/50 diet flew an average of 15557 ± 9619.2 s, whereas the flies fed the 40/60 diet flew an average of 11490 ± 4758.6 s. Flies were unable to regain flight when fed the mez and 5/95 diets. Flies flew an average flight distance of 4134.2 ± 2303.3 cm/s on the 25/75 diet (Figure 5).

Diet significantly affected flight speed in female black flies (F4,52=8.97, P <0.01). Flies were able to fly an average of 35.23 ± 13.50 cm/s on the fru diet and 27.13 ± 7.26 cm/s on the 95/5 diet. Flies fed the 50/50 diet flew an average of 24.96 ± 9.98 cm/s,
Table 1 – Wing measurement values (measured in mm, given as mean ± SD) for female black flies on each of the ten diet treatments (dH₂O, 25fru, fru, 95/5, 75/25, 50/50, 40/60, 25/75, 5/95 and mez). All diets used were a 50 % w/v sugar concentration except the 25 fructose diet, which is a 25 % w/v sugar concentration. There was no significant difference between any of the diet treatments with respect to wing length (F₉,₈₃=0.61, P=0.7874).

<table>
<thead>
<tr>
<th>Diet Type</th>
<th>Wing Measurements (mm ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>2.72 ± 0.13</td>
</tr>
<tr>
<td>25fru</td>
<td>2.72 ± 0.16</td>
</tr>
<tr>
<td>fru</td>
<td>2.73 ± 0.12</td>
</tr>
<tr>
<td>95/5</td>
<td>2.68 ± 0.11</td>
</tr>
<tr>
<td>75/25</td>
<td>2.67 ± 0.08</td>
</tr>
<tr>
<td>50/50</td>
<td>2.78 ± 0.10</td>
</tr>
<tr>
<td>40/60</td>
<td>2.75 ± 0.12</td>
</tr>
<tr>
<td>25/75</td>
<td>2.74 ± 0.11</td>
</tr>
<tr>
<td>5/95</td>
<td>2.71 ± 0.12</td>
</tr>
<tr>
<td>mez</td>
<td>2.72 ± 0.16</td>
</tr>
</tbody>
</table>
Figure 4. Average flight distances measured in meters (± SE) for each of the seven experimental diets. Flight distances were analyzed using a One-Way ANOVA ($F_{6,74}=20.52$, $P < 0.0001$) with a post hoc Tukey (HSD) comparison of means test. Values followed by the same letter are not significantly different from one another at $\alpha = 0.05$. The numbers above the bars represent the number of flies flown.
Figure 5. Average flight durations measured in seconds (± SE) for each of the seven experimental diets. Flight durations were analyzed using a One-Way ANOVA ($F_{6,74}=16.16$, $P < 0.0001$) with a post hoc Tukey (HSD) comparison of means test. Values followed by the same letter are not significantly different from one another at $\alpha =0.05$. The numbers above the bars represent the number of flies flown.
Figure 6. Average flight speeds measured in cm/s (± SE) for each of the five experimental diets. Flight speed values were analyzed using a One-Way ANOVA (F_{4,52}=8.97, P < 0.0001) with a post hoc Tukey (HSD) comparison of means test. Values followed by the same letter are not significantly different from one another at α =0.05. The numbers above the bars represent the number of flies flown.
whereas the flies fed the 40/60 diet flew an average of $15.31 \pm 5.59$ cm/s. Flies were unable to regain flight when fed the mez and 5/95 diets. Flies flew an average speed of $15.67 \pm 4.52$ cm/s on the 25/75 diet (Figure 6).

**Part Two: Sugar Digestion Experiment**

The sugars present in the crops and midguts of 417 female black flies were analyzed using Thin Layer Chromatography (TLC). Table 2 shows the sugars that were detected in 50% or greater of the 417 flies at the various time intervals. The individual results (sugars detected) of all flies are shown in Appendix 2.

**Effects of Sugar Type on Digestion**

No sugars were detected in the flies that were not fed any sugars, and they were omitted from the table. The complex sugars melezitose and stachyose were never completely digested, whereas the simpler sugars such as fructose and sucrose were converted to glucose. The mixture diets (fructose and melezitose) all showed a similar trend of melezitose never being fully broken down.

**DISCUSSION**

**Flight Distance, Speed and Duration Comparisons**

The dH2O diet acted as a negative control to ensure that the flight resulting after a sugar diet was the result of the ingested sugar and that the fly was unable to draw on any stored glycogen reserves. As the flies fed the dH2O diet were unable to regain flight, this
Table 2. The crops and midguts of 417 flies were dissected and analyzed using TLC analysis. This table shows the sugars (present in greater than 50% of the flies sampled) that were present at the various times post ingestion.

<table>
<thead>
<tr>
<th>Diet Type</th>
<th>Time Interval Post Ingestion</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min</td>
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</tr>
<tr>
<td>glu</td>
<td>glu</td>
<td>glu</td>
</tr>
<tr>
<td>suc</td>
<td>glu/suc</td>
<td>glu/suc</td>
</tr>
<tr>
<td>sta</td>
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</tr>
<tr>
<td>fru</td>
<td>glu/fru</td>
<td>glu/fru</td>
</tr>
<tr>
<td>95fru/5mez</td>
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</tr>
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<td>40fru/60mez</td>
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<td>glu/fru</td>
</tr>
<tr>
<td>25fru/75mez</td>
<td>fru/mez</td>
<td>fru/mez</td>
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<tr>
<td>5fru/95mez</td>
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<tr>
<td>mez</td>
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</table>
shows that any resulting flight was due to the ingested sugar. This is important as all test flies were beginning from the same physiological state.

I hypothesized that flies would be able to fly longer (in distance and duration) on the diets that contained melezitose in addition to fructose. This hypothesis was true, I found flight distance to be approximately two times longer in flies fed the 50/50 (3529.0 ± 1913.6 m) diet than on the fru (1919.8 ± 957.96 m) only diet. Flight duration was approximately three times longer in flies fed the 50/50 (15557.0 ± 9619.2 s) diet than on fru (5004.3 ± 2827.5 s) only diet. Flies that were fed the fru diet had the highest flight speed (35.23 ± 13.5 cm/s) when compared to all the diets. One general trend among the average flight speed values is that as the amount of melezitose increases, the average flight speed decreases.

Digestion of Various Sugars

I hypothesized that flies would not be able to digest melezitose, but that the addition of a simple sugar would aid in melezitose digestion. Yet even when fructose was added, melezitose was never completely digested. The only case in which melezitose was broken down was in the case of a long elapsed time period (2 and 3 hours). Black flies are able to digest and use complex sugars, such as melezitose. Being able to digest and/or utilize this sugar when it is presented by itself, however, is not necessary, as melezitose would always be encountered along with a simple sugar (fructose, glucose or sucrose) in honeydew. Furthermore, Downes and Dahlem (1987) postulated that feeding on homopteran honeydew could be an ancestral trait in the Diptera. If this is true, one would
expect that black flies would be able to use the sugars in honeydew, such as the trisaccharide melezitose.

Summary of Flight and Digestion Experiments

The digestion experiment shows that black flies can breakdown melezitose in the absence of a simple sugar (as shown in the digestion experiment, when melezitose was broken down at 2 and 3 hours). From the results of the flight mill experiment, the addition of the complex sugar, melezitose did aid in increasing flight distance and flight. However, the 50/50 diet is probably the optimal diet, as it produced the longest flight distances and durations.

In black flies, the simple sugars probably fuel the initial stages of flight, and once enough time has passed, the complex sugar is broken down and can be used. From this study there is no evidence to support the hypothesis that simple sugars aid in the breakdown of complex sugars. However, simple sugars are apparently necessary for flight, as they are responsible for fueling flight until the complex sugar can be broken down and used.

Conclusion

This research project focused on determining why honeydew sugars produce significantly longer flight distances and durations than nectar sugars, by investigating the effects of the individual sugars on flight performance and the digestion of the individual sugars.
This is the first study to demonstrate that the addition of the complex sugar melezitose resulted in increased flight distances and durations. The digestion experiment demonstrated that a long amount of time is needed for melezitose to be broken down. In summary, the complex sugar melezitose results in increased flight performance in female black flies when sufficient time has been provided for digestion to occur.

**Future Research**

In future studies it would be informative to look at the effects of nectar and honeydew meals (including all components of these two diets such as lipids and amino acids) on black fly flight performance and digestion.
General Discussion

Both floral nectar and homopteran honeydew serve as sugar sources for black flies. The sugars gained from feeding on these two sugar sources fuel flight and increase longevity. Although both of these are wild sugar sources for black flies, their differing sugar composition suggests that they may differentially affect various life history aspects.

Most longevity studies that have been conducted compare various diets by providing the diets *ad libitum*. However, in a previous study when the longevity of black flies maintained on nectar and honeydew sugars was compared *ad libitum*, no significant differences among the two diets were visible (Stanfield 2000). If there are differences between the effects of nectar and honeydew sugars on black fly longevity, then they would most likely be visible under conditions of limited diet. Under conditions of limited diet, differences were detected for females of *S. venustum*, however differences were not visible for either sex of *S. truncatum*. The biology of these two larvae might play an integral part in the interpretation of these results. *S. truncatum* occurs primarily at the outflow areas of streams, which are the most nutrient rich area. *S. venustum* on the other hand, does not exhibit such a location specificity. From understanding something as specific as larval location within a stream, differences in how diet affects longevity at the adult stage can be better explained. When a larva emerges with sufficient reserves (e.g., when the larva was positioned in an optimal location, *S. truncatum*), diet type does not differentially affect longevity. However, when a larva emerges with less than sufficient reserves (e.g., if the larva was not optimally positioned within a stream, *S. venustum*). This hypothesis supports the results that were generated from my study. One other explanation that is also possible and does not exclude the previous hypothesis is that
other components present in floral nectar and homopteran honeydew may have the ability to enhance the differences between the effects that these two sugar sources have on black fly longevity.

In addition to the effects that nectar and honeydew have on longevity, these diets also affect flight performance and digestion. In a previous study Stanfield (2000) showed that honeydew-fed flies were able to fly significantly longer in distance and duration than flies fed nectar. So with my study I wanted to answer the question of what in the honeydew diet was the cause behind the significantly longer flight distances and durations. It was determined through this flight mill study that the melezitose present in the 50/50 diet that was producing the significantly longer flights. The flies fed the 50/50 diet flew the longest in flight distance and duration. From the results of the flight mill study, as well as the digestion study, it shows that black flies are able to use melezitose however, it is necessary to also have a simple sugar present, that is easily digestible and usable.
References


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Yee, W.L. & Anderson, J.R. 1995. Tethered flight capabilities and survival of
Appendix 1

Sugar Percentages in the Artificial Nectar Diet

25 % fructose  
35 % glucose  
40 % sucrose  

Recipe of Artificial Nectar  
25 g of fructose  
35 g of glucose  
40 g of sucrose  
200 ml of distilled water  

Sugar Percentages in the Artificial Honeydew Diet

15 % fructose  
20 % glucose  
25 % sucrose  
40 % melezitose  

Recipe of Artificial Honeydew  
15 g of fructose  
20 g of glucose  
25 g of sucrose  
40 g of melezitose  
200 ml of distilled water
Appendix 2

Table - The breakdown sugars of 417 black flies fed various sugars and killed at the appropriate time intervals (i.e., 2, 5, 10, 15, 30, and 60 minutes). The sugars are noted and the letters represent the individual samples.

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