Influence of dietary nutrients on life history-related traits of black flies and mosquitoes

by

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A Thesis

submitted to the Department of Biological Sciences

in partial fulfillment of the requirements

for the degree of

Master of Science

August 2012

Brock University St. Catharines, Ontario

ABSTRACT

The sugar-feeding ecology of dipteran vectors has recently been targeted because it presents opportunities to inoculate common food sources for these dipterans with entomopathogenic bacteria as a means of controlling the population of host-seeking adult dipteran vectors. Whereas this approach to vector control holds some promise, differences in the nutrient composition and concentration in sugary food sources can influence the food selection pattern of dipteran vectors and potentially confound the outcomes of field trials on the efficacy of entomopathogenic bacteria as vector control agents. Further, nutrient components of bacteria-inoculated artificial diets may present unintended effects of extending the survivorship or fecundity of the target population and potentially render the whole approach counterproductive. The present study investigated the diet-specific factors that influence the foraging decisions of female *Simulium venustum/verecundum* (Diptera: Simuliidae) and female *Anopheles stephensi* (Diptera: Culicidae) on artificial nectar and honeydew. Paired choice experiments showed that the black flies forage more frequently from high calorie diets, which contained melezitose, or those diets that contained amino acids, compared to low calorie melezitose-free diets or amino acid-free diets. The mosquitoes however displayed a more random diet selection pattern. The effects of sugary diets on certain life-history traits considered to be important to the ecological fitness of the black flies and mosquitoes were also investigated. Sugary diets had no significant effect on the survivorship and fecundity of the black flies, but they influenced the resistance of Leucocytozoon-infected flies to the parasite. Amino acid-containing diets appeared to extend the survival of mosquitoes, and also allowed them to take more vertebrate blood when they blood fed.

ACKNOWLEDGEMENTS

Many thanks to the members of my supervisory committee past and present: Drs. Fiona Hunter, Jean Richardson, Alan Castle, John Middleton, Glenn Tattersall, and Vincenzo DeLuca. I am grateful for your insightful comments and support for this work.

I would also like to thank Dr. Joffre Mercier, for his encouragement and timely advice, which helped in no small way, to make this work possible. Sir, I have learned a lot from you and I am grateful for the opportunity to know you.

My deepest gratitude goes to Beulah Alexander and Kiel Ormerod, you two were my anchor when I was drifting away in confusion.

Finally, to my wife, Tina and daughter Michelle, thank you for the many laughs and lovely memories, I am blessed to have you in my life.

"Now to the King eternal, immortal, invisible, the only God, be honor and glory for ever and ever. Amen". Thank you Father for bringing me this far.

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CHAPTER 1

GENERAL INTRODUCTION

Foraging behaviour is important to the life of all heterotrophs. By being able to feed on certain food materials, organisms are able to maximize their energy intake. Biting flies such as black flies and mosquitoes feed on sugary food substances to obtain energy for growth and the maintenance of homeostasis. Previous works have shown that females of heamatophagous genera of both black flies and mosquitoes feed on sugary foods in addition to blood feeding (Burgin & Hunter, 1997a; Gary & Foster, 2004; Manda et al., 2007). Other works have shown that sugar-feeding influences flight performance and fecundity (Hazzard, 2003; Stanfield, 2003). What is unknown from current literature are the diet-specific factors that affect the diet selection pattern of insects and how these factors impact the effect of sugar feeding on traits that have important fitness consequences. The purpose of this thesis is to provide some understanding of the factors that influence the foraging decisions of female black flies and mosquitoes and how these factors impact the ecological fitness of the flies. Fitness has been traditionally defined as the relative rate of increase of a genotype in a population (Brown et al., 1993). In this definition, the sub-population with a particular fitness-related allele is the basic unit on which the forces of evolution act. Ecological fitness on the other hand, can be thought of as classical or Darwinian fitness, i.e., the lifetime reproductive success of an individual (Kozlowski, 1996), which is a product of survival and fecundity.

Evolutionary history of insect feeding

Feeding modes of adult stages of terrestrial insects may be broadly classified into two categories: solid feeding and fluid feeding. Solid feeders include herbivores that feed on plant tissue as well as carnivores that feed on other insects. Fluid feeders include those that feed on plant or phloem sap, nectar, algal and fungal protoplasm, vertebrate blood, and excretions and secretions from plants and animals.

By the carboniferous period, about 300 million years ago, almost all plant-feeding insects had evolved ways of utilizing plant tissue and plant derived organic compounds, such as nectar as food sources (Bernays, 1998). According to Foster (1995), nectar feeding did not occur until the cretaceous period when flowering plants appeared. Foster (1995) suggests that prior to the advent and rapid diversification of angiosperms in the cretaceous period, herbivorous insects may have depended on gymnosperms for their food supply. As an extension to the preceding argument, dipterans may have fed on homopteran honeydew or extra-floral nectars in the time leading up to the evolution of flowering plants. Thus, honeydew feeding may be a more ancient form of sugar feeding in dipterans.

Perhaps the most important structural evolution among plant-feeding insects is the modification of head appendages into mouthparts that are adapted to mainly biting, chewing or sucking of food materials. To feed on solid foods, insects evolved mandibulate mouthparts that allowed them to cut and chew through the structural bulk of plants to get the food material. Fluid feeders (including those insects that feed on phloem and xylem sap, exudates from plant and animal wounds) evolved elongate mouthparts that are adapted for both invasive and non-invasive fluid feeding (Labandeira, 1997).

These structures would later become useful when insects began exploring animal tissue such as blood for nutrient supply.

Sugar feeding

Honeydew and floral nectars probably make up the bulk of sugary food resources that are used by insects to satisfy their nutritional requirements. They are the most extensively studied food sources for insects and other animals in the wild, and studies that are meant to investigate their effect on some measure of insect fitness (such as fecundity, survivorship and parasite resistance) have used artificial prepared diets that mimic those available in the wild in the terms of nutrient composition and concentration (Baker, 1977; Blüthgen et al., 2004). It is often assumed in older literature, that floral and extra-floral nectars are the only sugary food sources for black flies and mosquitoes but recent records indicate that these dipterans utilize sap from plant wounds and homopteran honeydews as alternate sugary food sources. Further, recent literature suggest that nectar feeding may be less frequent than previously thought (Gary & Foster, 2004; Manda et al., 2007).

Sugar feeding occurs in all males and in females of some obligate autogenous species-those species that develop all their eggs without taking blood meals-where both have undeveloped or atrophied mandibles because biting mouthparts are not required for this feeding behaviour (Anderson, 1987). During sugar feeding, the labella draw up the sugar meal into the folded cuticle by capillary action and draw it into the crop by pumps (Sutcliffe & McIver, 1984). The sugar feeding patterns of female black flies and mosquitoes may be nonrandom, and because sugar feeding provides the energy for flight

necessary for locating a suitable blood meal source, these heamatophagous black flies and mosquitoes may forage for sugar before seeking blood.

Both sexes of black flies require sugar as a source of energy for flight and other metabolic needs (Currie & Adler, 2008). Nectar feeding is primarily derived from open flowers but may be augmented with sap feeding from plant wounds. Burgin and Hunter (1997a; 1997b) showed that black flies readily feed on homopteran honeydew. Common homopteran producers of honeydews used by blackflies include: white flies (Aleyrodidae), aphids (Aphididae and Adelgidae), leafhoppers (Cicadellidae) and gall coccids (Kermidae) (Adler et al., 2004). Nectar sources for most species are not well known (Adler et al., 2004), and whether these sources are located by sight or scent or whether species exhibit strict preference for some flowers is also unclear (Crosskey, 1990).

Nectars and honeydews are essential components of the adult mosquito diet and diets for many other disease vector species (Spencer et al., 2005). Female *Anopheles gambiae* typically sugar-feed (when sugars are available) before seeking blood (Gary & Foster, 2004). Both sexes readily take sugar meals within hours of emergence from the pupal case, and almost always within 1 – 2 days thereafter (Foster, 1995). Sugar feeding in both sexes follows a largely unimodal crepuscular or nocturnal diel rhythm, which is similar to that of flight and other activities (Gary & Foster, 2006; Williams & Kokkin, 2005). Satiation can be reached in less than a minute, but on dried sugar, mosquitoes may salivate to dilute the sugars and continue feeding, thus extending the feeding time. Although there is no record of mosquitoes feeding on honeydew in the wild, Gary and Foster (2004) state that sugar feeding in mosquitoes is not restricted to feeding only on

certain types of sugars. Mosquitoes are therefore expected to feed on honeydews when they are available.

Where oviposition sites are not readily available, mosquitoes take more frequent sugar meals (Gary & Foster, 2006) perhaps to increase their calorie intake as they repeatedly search for suitable sites to lay their eggs. Spencer et al. (2005) also found that during dry seasons, where there are prolonged periods of dryness and suitable oviposition sites are rare, female *Aedes* mosquitoes fed on plant-sugars more frequent than during rainy seasons. Therefore, availability of sugar meals can influence the reproductive success of female mosquitoes.

Nectar

Nectar is a sugary plant secretion that is derived from phloem sap (Blüthgen et al., 2004). Nectar is composed of a mixture of many different nutritional and non-nutritional substances. The most abundant component of nectar is water, followed by sugars such as glucose, sucrose and fructose; sometimes melibiose, maltose and raffinose have been found to be present too (Baker & Baker, 1977; Crosskey, 1990). It also contains variable amounts of amino acids, inorganic ions, organic acids and less frequently, lipids, phenolics, and alkaloids (Baker & Baker, 1986; Blüthgen et al., 2004; Carter et al., 2006; Chalcoff et al., 2006; Hunt et al., 1982).

Both blackflies and mosquitoes feed on nectar (Burgin & Hunter, 1997a; Manda et al., 2007). The only digestion needed is sucrose hydrolysis, as the monosaccharides and water are rapidly absorbed across the gut of insects (Nicolson, 1998). Floral nectars are produced by angiosperms as a reward to animal pollinators. Plant species from at least 66 families produce extra-floral nectar on their leaves or shoots, which function

presumably to attract feeding insects that in turn, defend them against herbivorous mammals, (Heil et al., 2001). Nectar feeding occurs late in the day, on flowers that are generally small, white and arranged in clusters (Adler et al., 2004; Crosskey, 1990). Whether nectar sources are located by sight or scent or whether species exhibit strict preference for some flowers is unclear (Crosskey, 1990).

Honeydew

Honeydew is a generic term for the liquid droplet excretion from the alimentary tract of sap-feeding homopterans (Auclair, 1963). Homopterans produce honeydew for two reasons: as a means of achieving water balance, and to attract ants that tend to protect them when they collect the honeydew (Fisher et al., 2002). Honeydew is usually found viscous or solid on leaves and on litter, and its distribution depends on the local distribution of the homopterans that produce it. Common homopteran sources of honeydew include white flies (Aleyrodidae), aphids (Aphididae and Adelgidae), leafhoppers (Cicadellidae) and gall coccids (Kermidae) (Hazzard, 2003). Honeydews are essentially of plant origin; however, in addition to plant-derived sugars such as glucose, fructose and sucrose, honeydews also have homopteran-synthesized sugars such as melezitose (Faria et al., 2008). Melezitose is a non-reducing trisaccharide, which can be partially hydrolyzed to glucose and turanose (Hembry et al., 2006). Amino acids such as asparagine, glutamine, isoleucine, tyrosine, lysine, histidine and arginine occur in honeydews in various varieties and quantities, depending on the age of the producing homopteran and whether they are tendered by ants or not (Sandstrom & Moran, 2001; Yao & Akimoto, 2002).

Honeydew composition differs between homopteran species. When tended by ants, aphids produce honeydew more frequently but in smaller droplets (Yao & Akimoto, 2002). The honeydew of the tansy aphid, *Metopeurum fuscoviride* feeding on tansy leaves was generally composed of glucose and fructose, sucrose, trehalose, and melezitose (Fischer et al., 2002). In the cottonseed bug, *Oxycarenus hyalinipennis,* complete digestion of melezitose occurs in the gut where transglycosidic invertase hydrolyses this trisaccharide to glucose, fructose and turanose (Bhatnager, 1964). Although the nutritional value of melezitose is lower than that of glucose, sucrose or fructose (Wäckers, 2000), it has been reported to attract ants and bees (Fischer & Shingleton, 2001) and may be able to attract mosquitoes and black flies to feed on wild honeydews.

Fitness benefits of sugary food feeding

Fitness is generally thought of as the suite of behavioral, physical and physiological adaptations that improve the reproductive success of the individuals that possess them relative to others in the population that do not. This way of thinking about fitness presents one major difficulty; the potential fitness benefit of any factor can only be studied in subsequent generations of the population under study. This approach disregards the benefit of the trait to the group of individuals that currently possess it. Peacock (2011) argues that fitness is most "usefully understood as those properties of organisms that are explanatory of survival in the broadest sense, not merely descriptive of reproductive success". Fitness is more appropriately viewed as an evolutionary ecology phenomenon where the evolutionary histories of individuals as well as the interactions between them and their environment influence their relative survival.

MacArthur & Pianka (1966) proposed that organisms would forage in such a way as to maximize their fitness. This fitness is usually measured in terms of energy intake Mitchell (1981). Sugars serve as some of the simplest, most easily metabolized primary energy source for many insects (Kent & Robinson, 2009; Wäckers et al., 2008). Sugars may enhance survival and fecundity and can even have a direct effect on insect resistance and resilience to parasites, diseases and injury (Molleman et al., 2008). Both sexes of *Anopheles gambiae* will die if they do not sugar-feed within the first 36 hours of emergence at 27°C (Foster & Taken, 2004). Nectar is known to enhance fecundity in some blackfly species, and both sexes in most species commonly feed on plant sugars soon after emergence and prior to mating (Anderson, 1987). Davies (1952) reported that sugar-fed female *S. venustum* flies lived longer than those provided with water only and the addition of albumin to sugars slightly increased the survival of the flies. Adult simuliids in the laboratory survive for only 1 - 2 days without a sugar meal (Rodriquez-Perez et al, 1995). Sugar feeding also improves the survivorship of both male and female mosquitoes (Gary & Foster, 2004). Mosquitoes that fed on honeydew were found to have fewer *Plasmodium* oocysts on their midguts (Hunter & Recce, unpublished data).

The amino acid and sugar components of sugar meals may contribute to their attractiveness to insects. According to Carter et al. (2006), insects have the ability to 'taste' proline, and it acts to elicit feeding responses from insects when they probe for nectar. Melezitose, the honeydew sugar, also enhances the gustatory response of moths, ants and bees to honeydew feeding (Winkler et al., 2005; Woodring et al., 2004). Sugar feeding provides energy for flight and survival and augments reproduction in mosquitoes (Foster, 1995). Nectar is known to enhance fecundity in some autogenous species, and both sexes in most species commonly feed on plant sugars soon after eclosion prior and

to mating (Anderson, 1987). Females of some species require sugars for ovarian development (Cupp & Collins, 1979) and in other species they permit autogeny (Corbet 1964; Hunter, 1977).

Insects that feed on certain types of sugar meals have been found to live longer than those that do not. Manda et al. (2007) reported that mosquitoes that fed on nectars with high sugar concentrations under natural conditions lived longer and laid more eggs. The diamondback moth parasitoid wasp, *Diadegma insulare,* lived in excess of two weeks when fed with buckwheat floral nectar, but lived only six to seven days when provided with soybean aphid honeydew (Lee et al., 2004). Lee et al. (2004) suggest that the extended longevity of nectar-fed wasps over honeydew-fed ones is due principally to the higher nutritional value of the nectar. Nutritional value is the net calories available to a consumer after the cost to obtain, ingest and process the food has been accounted for.

Often, the value of a food source to a consumer is measured in term of the total amount of calories contained in a unit of that food material. Mitchell (1981) argues that this approach only appropriately measures the potential value of food and that the actual value of any food material is its nutritional value, which depends on the chemical composition of the food source and the digestive physiology of the consumer. In the study by Lee et al. (2004), the honeydew contained melezitose, an oligosaccharide, which according to Wäckers (2000), the digestive system of this parasitoid is not equipped to digest. Despite the low nutritional value of honeydews, Hogervorst et al., (2008) reported that the larvae of the green lacewing *Chrysoperla carnea* preferentially fed on aphid honeydew even in the presence of high quality prey in order to extend its longevity. This observation introduces another variable in the assessment of the value of food material to a host, that it may be age-dependent.

Blood feeding

Heamatophagy or blood-feeding behavior evolved independently at least six times in about 14,000 – 15,000 species of 400 arthropod genera during the Cretaceous and Jurassic eras (145 – 65 MYA) (Graça-Souza et al., 2006; Ribeiro, 1995). Arthropods that lived in close association with vertebrates may have first fed on exfoliated skin or other vertebrate by-products. Later, with the evolution of piercing and sucking capabilities, these arthropods explored the more nutrient-rich vertebrate blood. In heamatophagous insects, blood provides the precursor amino acids needed for the synthesis of yolk in developing oocytes (Attardo et al., 2005). For this reason, blood-feeding behaviour proved to be advantageous to the reproductive fitness of populations of insects that utilized the resource and, therefore, was selected to form part of the feeding regimen of the heamatophagous insects. To reach blood vessels to extract vertebrate blood, blood-feeding insects cut or pierce the skin of their victims using various versions of the mandibulate mouthparts and an equally diverse method of sucking action by using the elongate mouthparts.

Blood feeding in black flies is thought to be a secondary adaptation aimed at improving the relatively poor nutritional prospects yielded by feeding on plant sap in pre-modern species (Crosskey, 1990). Females of most species require blood from endotherms to develop their eggs and show differences in their choice of vertebrate hosts. Crosskey (1990) identifies three sub-groups of such black flies. These include Ornithophilic flies, which feed on blood of birds, Mammalophilic flies, which feed on mammals except man, and, Anthropophilic flies that feed on human blood. Together, these blood-sucking black flies make up 90% of North American species of black flies, the other 10% being non-heamatophagous (Crosskey, 1990).

Biting flies have developed sensory perceptions that aid in the location of potential hosts. For daytime feeders, visual cues are important in locating potential hosts. Mosquitoes and black flies appear to prefer certain colours to land on, and large moving objects attract tsetse flies (Ribeiro, 1995). Heamatophagous insects are able to intercept chemical cues such as carbon dioxide, water vapour and lactic acid (common in human sweat) given off by the potential host (Shemanchuk, 1987). Host seeking begins with the dispersal or displacement of populations over wide areas to occupy host habitats (Shemanchuk, 1987). Some species may actively search for hosts, whereas other species may wait until hosts come within range of detection. Host location and subsequent feeding involve a series of steps in which habitat features and host specific attributes play a role in host selection (Adler et al., 2004).

Once close to a host however, the host's CO_2 emissions, colour and body movements are the most important factors that keep swarming females in close proximity until landing cues are received (Shemanchuk, 1987). The fly is ultimately attracted to specific parts of the host's body based on stimuli such as light, temperature, humidity, skin and hair colour and texture, and odours from whole blood (Wenk, 1987). Blood from fresh wounds attracts more females to wound areas and stimulates biting behaviour in others (Shemanchuk, 1987). Once a fly has landed on a host, muscle action supplemented by the fly's own body weight bends the labrum, stretching the victim's skin to allow for easy laceration by the snipping action of the mandibles (Sutcliffe & McIver, 1984), as blood oozes out from the wound, the fly laps it and deposits in the wound anti-coagulants from the salivary glands to prevent the blood from clotting. At this point, the fly may transmit to its victim any pathogen that it carries and, in turn, may pick up parasites from

host's peripheral blood. Ingested blood is stored in the midgut (Sutcliffe & McIver, 1984).

Medico-veterinary importance of biting flies

The vector potential of an insect is directly linked to the insect's life history and ecology. To be effective in vectoring a pathogen, a vector must pick up sufficient quantities of a parasite from an infected host's blood and be able to support the development or growth of the parasite. Blood-feeding behavior is probably the most important life trait contributing to the efficiency of insects as disease vectors. For many insects, blood feeding provides the proteins needed for egg development. The need to feed on blood is the reason why insects such as *Anopheles stephensi* (the Asian malaria mosquito) and *Simulium rugglesi* (Nicholson and Mickel) (the blackfly vector of *Leucocytozoon simondi* the causative agent for duck malaria Barrow et al., (1968) are of such medical and veterinary importance.

The blood feeding behaviour of various dipteran species harms humans and livestock in several ways. Aside from the nuisance caused by host-seeking females and males which may congregate around a vertebrate host in a bid to mate with females, the more serious repercussions of blood feeding such as disease transmission and allergic shock in response to vector-derived molecules are well documented. Black flies (Simuliidae) and mosquitoes (Culicidae) are among the most notorious blood feeders. Both these insect families contain species that are implicated worldwide as vectors of numerous arthropod-borne viruses and other parasitic disease-causing pathogens affecting human and animal health (Jones et al., 2004).

Black flies pose significant problems to human and animal health in many parts of the world because of the aggressive blood-feeding behaviour of some adult females. They are responsible for transmitting parasitic disease organisms, such as filarial worms, protozoa and arboviruses to man and a wide variety of domestic animals (Adler et al., 2004). Filarial worms are particularly important because species of *Onchocerca* parasitize both ungulates and humans. The causative agent of "river blindness" *O. volvulus*, is vectored by the *Simulium damnosum* complex in West Africa (Kurtak et. al., 1987). Infestation with this filarial worm can result in ocular and skin lesions, pruritus, insomnia, and general lack of energy with a concomitant reduction in socio-economic productivity of the infected individuals (Wogu & Okaka, 2008).

The veterinary problems posed by black fly bites may include death, morbidity, decreased meat and milk production, as well as reproductive dysfunction (Cupp, 1987). Several species of the parasitic protozoan *Leucocytozoon* are vectored by a few simuliid species to a variety of bird species. For example, *S. rugglesi* transmits *L. simondi* among ducks and geese, and *L. smithi,* which parasitizes turkey, is vectored by *S. slossonae*. In both cases, infection can result in high mortality levels among the bird populations (Adler et al., 2004). *S. vittatum* black flies transmit the Vesicular Stomatitis Virus New Jersey strain (VSV-NJ) to pigs (Mead et al., 1999). According to the center for Food Security and Public Health at Iowa State University (2008), VSV-NJ infection typically causes lesions on the feet, snout and udder of pigs and can render them lame and eventually kill them. Infected black flies can also transmit the virus to uninfected flies feeding in close proximity (Mead et al., 2004), thus increasing the chances of enzootic vesicular stomatitis within a drove of pigs.

Although mosquito bites can be painful and cause discomfort, the consequence of a mosquito bite can be far more severe than discomfort and itches. In many parts of the world, mosquito bites pose serious public health and socio-economic concerns. Mosquitoes are perhaps the most important insect vectors; they transmit disease-causing viruses and protozoa to humans and animals alike. Among the diseases transmitted by mosquitoes, malaria, dengue fever and West Nile fever are perhaps the most well known and best studied. Of these, malaria is the most important because of the morbidity and mortality rates associated with the disease. Malaria is a human disease caused by infection with protozoan parasites of the genus *Plasmodium*; it is by far the most important tropical parasitic disease. Malaria remains the single most important threat to juvenile survival in sub-Saharan Africa (Craig et al., 1999). In infants and non-immune individuals, infection with the falciparum species of the malaria parasite often results in severe anaemia and eventual mortality.

Malaria is arguably the most important challenge to the economic development of most countries in the tropics; it is the leading cause of poverty and low economic productivity in countries where the disease is endemic (World Malaria Report, 2003). In Ghana, West Africa, for example, malaria morbidity accounts for 40% of outpatient attendance to health facilities, and it is the number one cause of workdays lost due to illness, thereby contributing more to potential income lost than any other disease (Asante & Asenso-Okyere, 2003).

Veterinary importance of mosquitoes is largely restricted to the disease they transmit to poultry. Avian malaria is a disease caused by Apicomplexan parasites such as *Plasmodium*, which is transmitted by some *Culex* mosquitoes. Avian malaria has a worldwide distribution and is of great economic significance to the poultry industry,

where an infection can cause up to 90% mortality in poultry (Williams, 2005). Infected birds are often weak, anaemic, anorexic, depressed, and show significant weight loss when parasite burdens are high (Williams, 2005).

Vector-parasite interactions and effects of sugar feeding

Successful transmission of parasites depends on the coevolution of a stable strategy that balances the harmful effects of parasite virulence on the vector organism with the resistance of the vector to the parasite. Since a parasite extracts resources from the vector organism, the survival of the vector is central to the survival of the parasite. However, parasite proteins, being foreign to a vector's immune system, will ultimately elicit some form of immune response from the vector or at least a basic modification in the behaviour of the vector to rid itself of the parasite. The extent to which a vector invests in biochemical or behavioral means to rid itself of the parasite will depend on the cost associated with transmitting the parasite. This dynamic has both ecological and evolutionary implications. Primitive and relatively inexpensive forms of defense could lead to the evolution of elaborate suites of neural and biochemical functions that can prove to be effective at removing the parasites but extremely expensive (energetically) to the vector organism.

Where a resistance mechanism requires energetic or nutritional input, the diet of the vector will play a significant role in the successful implementation of that mechanism. Rodti and Lehane (2008) observed that tsetse flies that rid themselves of trypanosomes showed increased energy usage. From this observation, it can be predicted that intake of dietary calories will improve the energy budget of dipteran vectors and increase the likelihood that they would be able to rid themselves off their parasites or at least limit the

parasite load. High calorie diets may enhance the ability of vectors to limit the proliferation of parasites and therefore keep parasite load at levels that are less harmful.

Apart from energetic cost, insect vectors may incur other costs such as tissue loss. Baton and Ranford-Cartwright (2007) showed that the invasion of midgut epithelium of *Anopheles stephensi* by *Plasmodium falciparum* ookinetes caused the destruction and irreversible loss of midgut epithelium and parasite-invaded epithelial cells. Since an intact midgut wall is necessary for successful digestion of blood (Billingsley, 1990), and blood feeding is required for vitellogenesis in some dipteran vectors, heamatophagous insects will be expected to evolve some form of mechanism that allows them to reap the benefits of blood feeding while lowing the associated risks.

Desser and Yang (1973) suggested that the survival of black flies and biting midges is inversely related to the intensity of parasitemia in the birds they fed on. A prediction from this hypothesis is that biting flies should feed less frequently on a parasite-infected blood host. Tomas et al. (2008) supported this hypothesis by showing that black flies preferentially attacked hosts whose parasitemias had been reduced by medication, while avoiding those hosts whose level of peripheral blood parasite intensity were high.

Parasites may modify the behaviour of their hosts or vectors. This has been described as a parasite-driven strategy to ensure successful parasite multiplication and transmission (Holmstad et al., 2006). Such behavioural modifications may result in fitness costs to the host or vector. Rivero and Fergusson (2003) found an increased frequency in sugar feeding in *Plasmodium* infected mosquitoes. Perhaps the parasites modify the behaviour of the vectors to feed more on sugars and, therefore, indirectly ensure that the vectors acquire enough resources, which they can use for their own

development. However, glutamine, which is common to both nectars and honeydews, is converted to γ-aminobutyric acid (GABA) and is known to have antifeedant properties in insects (Shelp et al., 2006). For this reason, nectar or honeydew feeding may reduce the tendency to take blood or the amount of blood taken by a vector and, hence, limit the number of blood stages of a parasite that a vector will pick.

Components of some sugary diets are precursors for the manufacturing of important immune molecules. The insect gut epithelium lacks the mucus layer of vertebrates. Instead, it is protected physically by a chitinized peritrophic matrix (Rodti & Lehane, 2008), which is further protected by reactive oxygen species (ROS). One such ROS is nitric oxide, which *An. stephensi* produces to limits the growth of *Plasmodium berghei* parasites in the midgut (Akman-Anderson et al., 2007). Nitric oxide is synthesized from arginine, an amino acid common to nectars and honeydews (Yao & Akimoto, 2002). It should be expected, therefore, that feeding on nectars and honeydews would supply this precursor molecule, which could be useful in parasite resistance.

Digestive physiology of dipterans

The diet of adult dipterans is composed of carbohydrates and proteins that must be digested for subsequent absorption and assimilation. Many insects are equipped with appropriate physical and biochemical capabilities to make digestion possible. Just like in many other animals, digestion in dipterans occurs throughout the entire length of the alimentary tract. In dipterans that feed on plant-derived fluids and vertebrate blood, such as black flies and mosquitoes, food type determines the destination and the site of digestion of ingested food (Friend, 1978). In mosquitoes for example, ingested sugary solutions (especially those with high osmotic pressure) are temporarily stored in the crop

and passed on in small amounts to the midgut for digestion, whereas ingested blood goes directly to the midgut to be digested (Friend, 1978). The walls of the crop are impermeable to water and offer a barrier between the high osmotic sugary diet and the haemolymph (Nicolson, 1998).

Digestion in insects occurs in three phases. First, polymer hydrolases such as trypsin and amylase hydrolyze polymers into oligomers, which undergo further hydrolysis by oligomer hydrolases such as amino-peptidases and amylases to produce dimers, which are finally split into monomers by dimer hydrolases such as dipeptidases and maltase (Terra, 1990). The monosaccharide products of carbohydrate digestion are then passively absorbed through the midgut wall being facilitated by the high osmotic content of the midgut coupled with the conversion of glucose in haemolymph to trehalose (Nicolson, 1998). However, some polymers escape digestion in the digestive tract and enter the insect's haemolymph. Undigested proteins of the host organism have been found in the haemolymph of several *Anopheles* species after blood feeding (Jeffers & Roe, 2008). Movement of undigested proteins across the midgut wall is not unique to blood-feeing dipterans, as it has also been shown to occur in other dipterans as well, one example being the flesh fly, *Sarcophaga falculata* (Zlotkin et al., 1992).

The salivary glands of the female mosquitoes contain α-glucosidase, which breaks down starch and disaccharides to glucose units in their proximal lobes, and an apyrase, which hydrolyzes ATP to produce energy in the distal lobes. When taking a sucrose meal, mosquitoes selectively secrete α-glucosidase, but when blood feeding, mosquitoes secrete both α-glucosidase and apyrase (Marinotti et al., 1990). The salivary glands of some dipterans produce amylases, thus making starch hydrolysis possible even

when the insect is ingesting the food (Fereire et al., 1993). Starch digestion continues while the food is being stored in the crop (Terra, 1990).

Nectars and honeydews often exist in solution form in the wild and as such are expected to exert some osmoregulatory costs on the insects that feed on them. In the current study all diets are given in the solution form, with simple components at different concentrations. Thus, when test subjects ingest the diets; the nutrients would be readily absorbed leaving a balance of water. Whereas nectars (and honeydews) are osmotically concentrated, its consumption can lead to water excess (Nicolson, 1998) that must be regulated. In insects, the hindgut and the malpighian tubules are responsible for osmoregulation. Malpighian tubules are single celled tubular structures that open into the alimentary canal, arise at the junction of the midgut and hindgut, and blind-end in the haemolymph. The distal portion of the tubule (relative to the digestive tract) secretes fluid into the lumen along with other waste products; the distal portion and hindgut reabsorb water and essential solutes (Beyenbach & Piermarini, 2011).

Foraging theory

Insects, like all other heterotrophs, need to acquire energy (to grow and to maintain homeostasis) from consuming other organisms. Usually, a forager expends energy while foraging for food and must decide, therefore, how much energy needs to be invested in order to obtain the energy locked up within the tissues of the food substance. MacArthur and Pianka (1966) proposed the Optimal Foraging Theory to describe the foraging behaviour of heterotrophs. Basically, the model predicts that foragers will feed from food sources in such a way that they maximize their energy intake per unit of foraging time. The energy obtained from feeding on a food source expressed as a ratio of

the time required to locate, feed on, and digest the food, is referred to as the profitability of that food source (MacArthur & Pianka, 1966). Therefore, a consumer can be described to be foraging optimally if it feeds on the most profitable food source available to it.

Foraging theories assume that existing feeding behaviours have been shaped by natural selection and that they are close to optimal (Brown, 1993). Evolutionarily, a feeding behaviour becomes important if it results in a direct or an indirect increase in the fitness (or some estimate of it) of the population in question. The fundamental assumption employed in inferring the evolutionary importance of a foraging behavior is that fitness increases with energy gain.

Brown (1993) classified optimal foraging theory models into two broad categories. The first is the dynamic optimization model, which predicts the foraging decisions of consumers under typical natural circumstances, where they have to feed from food sources that are not always readily available in the immediate vicinity of the consumer and require different levels of energy investment to forage from them. The second category is the static optimization model. It assumes that there is no variability in the distribution in time and space of the various food substances available to a forager, and, therefore, there is no difference in the amount of energy that needs to be expended to forage from those food sources.

In a static situation, the category after which diet selection experiments in this research was modeled, time to locate a food source and feed from it is assumed (by experimental design) to be constant for each diet from which flies could selectively feed it is also assumed that this time is no different from the time required to locate and feed from the alternative food source. In this instance, the profitability of a diet will depend on the total energy content and the digestibility of the nutrients contained in the food.

Although diets used in the present study consist of free nutrients not bound to the structural bulk of some organism's tissue, differences in the chemical structure of the various nutrients may present different challenges to the digestive physiology of black flies and mosquitoes. Therefore, total calories available in a unit quantity of a diet may not necessarily be the most important factor in the foraging decisions of the black flies and mosquitoes under the experimental conditions of this study. Given the differences in the digestibility of diets (due to the component nutrients), black flies and mosquitoes may not forage in a manner predicted by McArthur and Pianka's (1966) model of optimal foraging. Rather, they may selectively feed from diets that are commonly more available in the natural environment. Hence, the rate at which an individual is able to extract the calories contained in an ingested food will be the principal predictor of the individual's diet choice.

Dietary self-selection

Dietary self-selection is broadly defined as the ability of foragers to discriminately feed on food sources in such a way that they obtain the most energy from the variety of foods available. The concept of dietary self-selection proposes that the foraging behavior of animals is nonrandom, and that there are benefits to discriminately feeding from certain foods. Learning can be important in the diet selection process. Bernays and Wrubel (1985) showed that grasshoppers learn to associate odors and colors with the nutritional quality of food and reject foods that had provided a poor nutrient balance. Different food types with different nutritional qualities occur in the natural environment of black flies and mosquitoes. It is reasonable to expect these insects to

display nonrandom feeding patterns and perhaps exhibit a pattern similar to those observed in grasshoppers by Bernays and Wrubel (1985).

Dipterans have also been known to discriminate between macronutrients. The chemosensory hairs on the labellum of the blowfly *Phormia regina* (a model organism for studies on insect gustatory response) have been studied extensively and have been shown to be responsive to solutions of glucose, fructose, sucrose and melezitose (Dethier, 1955). These sugars are exactly the same ones in the artificial diets used in the present study and though studies on the responsiveness of chemosensory hairs of Simullids and Culicids to the sugars these sugars are lacking, it is reasonable to predict that it would be similar to that of *Phormia regina*. Dethier (1961) indicated from his behavioral and electrophysiological experiments that the blowfly *(Phormia regina)* could discriminate among water, sucrose, and protein, respectively, and that these discriminations, in part, could be performed at the receptor level of the chemosensory cell of the legs and the mouthpart.

Research objectives

It is generally accepted that black flies and mosquitoes feed on floral nectar and/or homopteran honeydew for energy for metabolic requirements and the maintenance of homeostasis. Females of some black fly and mosquito species require vertebrate blood meals to be able to produce mature eggs. Thus, these two forms of feeding are important to the fitness of both insect families. Blood feeding is also important to an insect vector's ability to transmit pathogens because it is this behaviour that allows them to pick up or deposit pathogens into a host's blood stream. I suggest that in the wild, amino acid and sugar profile, and total sugar concentration of sugary diets will interfere with successful

blood feeding and potentially limit the effectiveness of vectors to transmit blood-borne parasites. Sugar feeding might interfere with blood feeding in a number of ways. First, the volume of sugar diet taken may limit how much blood may be subsequently ingested since the crop and midgut share the same abdominal space, and a replete sugary meal will exclude the possibility of a replete blood meal (Ma, 2010). Second, prior sugar feeding may reduce the flight performance of flies, in the natural environments as they have to compensate for the extra weight from sugar feeding and, thus, reduce their chances of finding a host from which to take a blood meal. Lastly, nectars and honeydews contain certain components that may have antifeedant properties and, thus, prevent vectors from further feeding (on blood or any other diet) after they had fed on those diets.

Given the medical and veterinary importance of black flies and mosquitoes, it is necessary to investigate the differential effects of sugar diets on life history traits that are important to their ecological fitness. The fact of mutual exclusivity of full sugary meals and full blood meals (Ma, 2010) can be exploited as means to reduce the entomological inoculation rate (EIR), of disease vectors in a locality. EIR is a measure of the local endemicity of an insect-borne disease per unit time, and includes the incidence of pathogen infection in the population of insect vectors (Shaukat et al., 2010). A reduction in the capacity of sugary-fed insects to blood feed to repletion indicates a reduction in the probability that pathogens will be picked up when the insect sugar feeds. *An. gambiae* have been reported to seek out sugar meals before searching for blood hosts (Gary & Foster, 2004). Whatever the underlying mechanism for this observation, feeding on sugary diets and blood feeding has been established to co-occur in mosquitoes especially.

The objective of this research is two-fold: to determine whether black flies and mosquitoes selectively feed on certain sugary food preparations, and to investigate the

potential benefits of selective feeding to common black fly and mosquito life history-related traits. From these objectives and based on current literature, I hypothesize that female *Anopheles stephensi* and female *Simulium venustum/verecundum* are able to discriminately feed from sugary food sources and that their diet selection pattern is determined by the total calorie content and nutrient composition of sugary diets. This hypothesis predicts that when given a choice, the mosquitoes and black flies will selectively feed from those diets with higher calorie content and greater diversity of nutrients. With regards to the fitness benefits of selective feeding, I hypothesize that total calorie content and nutrient composition of sugary diets influence fitness related life-history traits of *Anopheles stephensi,* female *Simulium venustum/verecundum,* and female *S. rugglesi*. I consider the amount of blood ingested, fecundity, parasite load and survivorship of as traits that are essential to the life of dipteran vectors and use them as indirect measures of biological fitness. From the hypothesis, I predict that flies that feed on high calorie amino acid-containing diets will take less blood, lay more eggs, have fewer parasites in midgut and live longer.

CHAPTER 2:

GENERAL METHODS

Care and use of host organisms

Care of ducks

Day-old Peking ducklings, *Anas bochas,* were obtained from Frey's Hatchery, St. Jacobs, ON and were transported to the Wildlife Research Station (WRS) in Algonquin Provincial Park. This location is known to have a population of *Leucocytozoon simondi*-infected wild ducks and an abundance of the *Simulium rugglesi* vectors. The ducklings were used as sentinel hosts from which blood-fed and infected female *S. rugglesi* black flies were collected.

The ducklings were kept in an animal holding facility and were provided with heat and light by a heat lamp in a 90 cm X 40 cm plastic cage and were provided with Shurgain® duck starter feed and water *ad libitum* as well as straw for bedding. Colored plastic O-rings were used as leg bands to identify each individual duckling. At about 2 weeks of age, the ducklings were fed Shurgain® meat builder and were transferred to an outdoor chain-link pen. They were housed in a 2 m X 1 m X 0.5 m chicken wire cage to protect them from predatory mammals such as weasels (*Mustela* spp.) and martens (*Martes* sp.), which had been previously reported to be able to go through the chain-link. None of the duck feeds used in this study contained any antibiotics. Routine care and handling of ducks were in accordance with animal care and use protocol (AUPP #08-05-01 and #10-04-02), as approved by Brock University's Animal Care and Use committee.

Natural infection of ducks with L. simondi sporozoites

At approximately 3 weeks of age, the ducks were taken for a one-hour swim in the Sasajewun Lake, which was about 30 meters from the chain-link pen. The purpose of this was to expose the ducks to infective bites from the *L. simondi* vectors, *S. rugglesi,* which had been reported to bite ducks more frequently at this location than any other within the WRS (Hunter et al., 1993). During swim sessions, the ducks were kept in a 2 m X 1 m X 0.5 m chicken wire cage to protect them from predators that may have been present in the lake.

Assessment of infection level of L. simondi in ducks

Ten days after being taken out for an exposure swim, a sterile lancet was used to puncture the femoral artery of a duck to collect blood samples with a heparinized microcapillary tube (Gill & Paperna, 2005). A thin blood smear was made on a clean microscope slide and was immediately air-dried, fixed with absolute methanol and stained with Diff-Quik® Stain Set (Imeb Inc.). The slide was then permanently mounted with Permount® (Fisher Scientific) and inspected under a light microscope (100X, oil immersion) for round and elongate gametocytes of the *L. simondi* parasite. The number of infected erythrocytes per 1000 erythrocytes was recorded as infection intensity. Ducks showing an infection intensity of 6-7% were used as a sentinel hosts to collect infected *S. rugglesi* (refer to section on *Simulium rugglesi* "colony" below).

Care of mice

Female CD-1 mice, *Mus musculus,* were obtained from Charles River Canada. Routine care and handling of mice were in accordance with animal care and use protocol (AUPP #10-05-04), as approved by Brock University's Animal Care and Use committee.

Infection of naïve mice with P. berghei ANKA strain gametocytes

The rodent malaria parasite *Plasmodium berghei* (MRA-311, MR4, ATCC Manassas Virginia) was obtained as gametocytes from the supplier. Upon arrival, the vial containing the frozen parasite-infected blood was thawed at room temperature. Three mice were randomly selected, weighed and anesthetized by intra-peritoneal injection of a ketamine-xylazine-distilled water mixture at a dosage of 0.1mL/10g of body weight. The content of the thawed vial was then injected into the anesthetized mouse via an intra-peritoneal route at a dosage of 0.1mL/20g body weight (Methods in Malaria Research, MR4). Beginning 3-4 days post infection, infection levels were monitored daily in the mice by making a thin blood smear from tail blood. The procedure for making a thin blood smear was the same as that described for the ducks.

Infection intensity per mouse was determined as the ratio of the number of parasitized erythrocytes per 1000 erythrocytes at 100X oil immersion. Infected mice with infection levels between 6-7% were used in blood feeding trials on the same day the infection intensity was determined to infect *A. stephensi* (Methods in Malaria Research, MR4). Parasites were maintained in the mouse "colony" by serial blood passage from an infected host to a naïve host.

Experimental infection of An. stephensi with P. berghei

Female mosquitoes were prepared for infective blood feeding by being denied access to sugar meals for up to 12 hours before the blood meal. Sugar-starved mosquitoes were transferred to 30cm X 30cm X 30cm cages for blood feeding. An infected mouse was anesthetized as described above and the abdomen was shaved to facilitate easy

access by mosquitoes for feeding. The mouse was placed abdomen-down, with the four limbs stretched out, on top of the mesh portion of the cage. The mosquitoes were allowed to feed for 20 minutes and were then transferred back into their holding containers.

Care and maintenance of fly 'colonies'

Anopheles stephensi 'colony'

An. stephensi Liston (wild type STE2, obtained from MR4) were reared at 27± 2°C, ~80% relative humidity and 14:10h (L:D) photoperiod in a constant environment incubator. All larval stages were kept in petri dishes at a density of 1 larva per mL of dechlorinated tap water and were fed daily with ground Koi® fish food. Pupae were harvested and transferred to one of two 30cm X 30cm X 30cm fine mesh cages. One of the cages, the 'stock colony', was maintained as described above. The 'stock colony' provided pupae used to start the 'experimental colonies'. Female adults of the stock colony were fed 10% sucrose *ad libitum* and were provided sheep's blood obtained from Cederlane® Laboratories Ltd. every fifth day to ensure a steady supply of progeny to maintain the 'colony'.

Blood meals were provided through Parafilm® 'M' stretched over the rim of a glass feeder. The glass feeder had a hollow interior, and by running hot tap water through it, the blood meals were kept at 38-40˚C throughout the 20-minute period when mosquitoes were taking blood meals. Blood-feeding procedures were performed in accordance with Brock University's Biosafety Protocol. General colony maintenance practices were in accordance with *"Methods in Anopheles Research Manual",* published by the Malaria Research and Reference Reagent Resource Center (MR4).

Simulium rugglesi 'colony'

Ducks with an infection intensity of 6-7% were used as bait-hosts to collect *L. simondi* infected *S. rugglesi* (Steele et al., 1992). Four ducks were individually confined to a 35 cm cube metal mesh cage on two 180 cm X 40 cm white boards placed on the beach of Lake Sasajewun. This procedure was performed just before dusk, which is the peak biting period for Simuliids. An exposure period of 15 minutes was allowed for blackflies to be attracted to the ducks. A drop cage, made of cloth mesh netting around a wooden frame, was placed over each metal cage after the initial 15-minute exposure period. Another 15-minute period was allowed for black flies to fully engorge and emerge from the plumage of the ducks. At this stage, the blood-fed flies resting on the inside of the drop cages were aspirated into small cages and were transported to the laboratory. It was assumed that visual cues, coupled with chemical cues from carbon dioxide and uropygial gland secretions from the ducks, were the only attractants used by *S. rugglesi* to locate the ducks, and that experimenter-manipulation did not affect the attractiveness of ducks to the flies.

Simulium venustum/verecundum 'colony'

Rock pebbles, suspended vegetation and other floating organic matter in Costello Creek (45° 35' 57.97'' N, 78° 19' 48.67'' W) were inspected for attached black fly pupae. Substrates with pupae were transported to the laboratory together with water collected from the creek. The substrate materials and water were transferred into plastic containers measuring 31 cm X 26 cm X 8 cm and were kept in emergence cages. The emergence cages were made from wood frames, covered with fine screen netting. Temperature inside the emergence cages ranged between 15 and 24 °C (average 20°C).

To mimic some conditions typical of the natural pupal habitats, an electronic Airtech 2K4™ air pump was used to create bubbles in each plastic container. This ensured an oxygen-rich environment and provided steady water current within the plastic container.

Emergence cages were inspected at six-hour intervals to collect emerged adults. This was done to ensure that age differences between adults used in the experiments were minimal. Emerged adults were visually inspected, and females with the characteristic yellowish-white patches on the front fore tibiae were aspirated and used in experiments. After each experiment, the flies were properly identified using the keys of Davies et al. (1962). Only flies belonging to the *Simulium venustum/verecundum* species complex were included in data analyses. Except where indicated, adult black flies were kept in an incubator with the following conditions: temperature 20 ± 2°C, photoperiod, 12:12 (L:D) and ~ 70% relative humidity.

Diets used in this study

Recipe of diets used in the experiments in the present study is found in Table. 2.1. Six types of sugary diets were made based roughly on the concentration of common sugars and amino acids found in natural nectars and honeydews (Carter et al., 2006; Chalcoff et al., 2006; Fischer & Shingleton, 2001; Fischer et al., 2002; Heil et al., 2000; Hogervorst et al., 2006; Petanidou et al., 2006; Woodring et al., 2004; Yao & Akimoto, 2002). There were three nectar diets and three honeydew diets each of which contained D-glucose, D-fructose and D-sucrose. D-melezitose was in the honeydew diets only. Concentrated nectar (CN) and concentrated honeydew (CH) contained the most solutes per volume of solution in each diet category. The diluted versions of these diets were

called nectar (N) and honeydew (H) respectively. L-asparagine and L-glutamine were added to each of the dilute diets to form what was called enriched nectar (EN) and enriched honeydew (respectively). Nectar diets were made with 5mL of 1% (w/v) of 4-amenobenzoic acid (PABA) (for preservation) and brought to a total volume of 100mL in distilled water. The honeydew diets were made with 2mL of 1% (w/v) of 4-amenobenzoic acid and brought to a total volume of 40mL in distilled. Therefore both diet types had a final PABA concentration of 0.05%. Total calorie content of each diet per mL was calculated based on reported values of calorie per gram of each component (Table 2.2).

Table 2.1: Recipe of diets use in this study.

	N	**H**	**EN**	**EH**	**CN**	**CH**
Glucose	1.45g	1.3g	1.45g	1.3g	3.5g	1.5g
Fructose	1.5g	0.85g	1.5g	0.85g	2.5g	2.25g
Sucrose	1.75g	1.12g	1.75g	1.12g	4.0g	1.0g
Melezitose	0	1.35g	0	1.35g	0	2.0g
Asparagine	0	0	2.6mg	1.1mg	0	0
Glutamine	0	0	5.1mg	2.1mg	0	0
Total solution vol. (mL)	100	40	100	40	100	40
Total Calories (kcal/mL)	0.18	0.45	0.18	0.45	0.38	0.65

Table 2.2: Caloric value of dietary nutrients.

Nutrient	kcal/g (Reference)
D-Glucose	3.75 (FOA, Corporate Doc. Depository)
D-Fructose	3.75 (Cat. Handbook of Physics & Chemistry)
D-Sucrose	3.94 (FOA, Corporate Doc. Depository)
D-Melezitose	4.05 (Cat. Handbook of Physics & Chemistry)
L-Arginine	5.35 (Voris, 1971)
L-Asparagine	3.42 (Voris, 1971)
L-Glutamine	4.21 (Tsuzuki & Hunt, 1957)

CHAPTER 3:

INFLUENCE OF SUGARY DIETS ON BLACK FLY LIFE HISTORY-RELATED TRAITS

INTRODUCTION

Black flies (Simuliidae) are a relatively small and structurally homogenous family of diptera (Currie & Adler, 2008) who are intimately bound to flowing freshwater (Adler et al., 2004). The immature stages (egg, larva, and pupa) all develop in fresh running water (Rubtsov, 1989). Adult body length ranges from 1.2 mm to 6.0 mm (Crosskey, 1990). Adult females, although having an entirely non-aquatic lifestyle, require running water to deposit their eggs. Black flies are cosmopolitan in distribution; they occur almost everywhere except Antarctica, some deserts and islands that do not have running water (Crosskey, 1990).

Adult black flies, males and females alike, require plant sugars, namely supplied from nectar and honeydew (Burgin & Hunter, 1997a) as a source of energy for flight and other metabolic needs. Adult females however, are best known for their blood sucking behaviour (Currie & Adler, 2008). This behaviour is essential in some species for the development of eggs. Some species however, have feebly developed mouthparts and are unable to cut flesh to take blood meals (Crosskey, 1990). These species, described as being obligately autogenous, emerge from the pupal stage with fully developed eggs (Currie & Adler, 2008). Other species must take at least a blood meal to develop their eggs. For these species, access to sugar meals may interfere with how much blood they can take and consequently, the number of eggs they are able to mature.

Simulium venustum/verecundum complex

Simulium venustum and *Simulium verecundum* are described as being sibling species. Adults of these species look alike morphologically and identification using morphological keys is unreliable. Alternative means of identification such as the use of polytene chromosome banding pattern of larvae (Rothfels et al., 1978) or by mitochondrial DNA sequences (Xiong & Kocher, 1993) have been developed to improve the accuracy of identification of the siblings in this complex. However, where there is limited access to the alternative identification tools, researchers routinely use morphological keys to distinguish this species complex from other simuliids (Davies & Peterson, 1956).

The *Simulium venustum/verecundum* is a mammalophilic species complex, which includes the principal noxious biter of humans on the Canadian Shield in the summer (Rothfels et al., 1978). This species complex is frequently the most abundant adult black fly species during late May and early June (the peak emergence season of black flies) at the Wildlife Research station, Algonquin Provincial Park, Ontario, Canada (Smith & Friend, 1982).

Simulium rugglesi

S. rugglesi is an ornithophilic species of blood-feeding black flies that frequently bite waterfowl and prefer ducklings and ducks over other birds (Barrow et al., 1968). Like other haematophagous black flies *S. rugglesi* are pool feeders; they penetrate the skin and produce small craterous lesions using a slashing or biting action involving the stylets and labium (Sutcliffe & McIver, 1984). This species is the most prevalent black fly feeding on waterfowl at the Algonquin Provincial Park, Ontario, Canada (Bennett,

1960). *S. rugglesi* transmits *Leucocytozoon* parasites among summer resident birds in Michigan (Barrow et al. 1968). The most prominent feeding period of the vector coincides with the period of elevated parasitemia in the peripheral blood of the avian host (Roller & Desser, 1973). The fly feeds for up to 12 minutes on the vertebrate host, and takes an average of 1.9 mm^3 of blood during that period (Bennett, 1963; Fallis, 1964). Typically, *S. rugglesi* return to the same site where they successfully took their last blood meal 5-7 days afterwards for another meal (Bennett, 1963).

Leucocytozoon spp.

Leucocytozoon protozoa are blood parasites of birds that produce a malaria-like disease known as "Leucocytozoonosis" (Adler et al., 2004). *Leucocytozoon* protozoa are prevalent in Simuliids; for example, 90% - 100% of ornithophilic species in the Algonquin Provincial Park, Ontario, have sporozoites in their salivary glands during the summer (Bennett & Squires-parsons, 1992). *Leucocytozoon spp*. cause infections in about 15 bird orders (Fallis et al., 1964). Infection with *L. simondi* may or may not be pathogenic and morbidity and mortality may also vary from year to year, with pathogenicity being severest in young birds (Ballwebber, 2004). Symptoms of the disease may include loss of appetite, emaciation, drowsiness, enlarged spleen, damaged liver, congested lungs and heart (Harwood & James, 1979) and convulsions that quickly lead to death (Adler et al., 2004). Chronically infected birds experience reduced reproductive abilities, depressed immune system and serve as reservoirs (Adler et al., 2004). Herman et al. (1975) implicated *L. simondi* as the prime cause of gosling deaths in populations of Canada geese in Seney National Wildlife Refuge in Michigan. Mortality levels in some duck and turkey operations range from 5% to 100% (Fallis et al., 1951).

Rationale and Research objectives

That there is heterogeneity in the sugary food sources available to dipterans in the wild has been well established. Indeed nectars and honeydews show diversity in nutrient composition and concentration, and the inclusion or otherwise of non-nutrient compounds (Baker & Baker, 1977; 1986; Blüthgen et al., 2004; Carter et al., 2006; Chalcoff et al., 2006; Fischer et al., 2002; Hunt et al., 1982; Yao & Akimoto, 2002). Burgin (1996) reported that sugars obtained by simuliids from plant and homopteran sources appear in as many as 16 different combinations or profiles in the black flies when assessed via thin-layer chromatography even in individuals of the same species. Several studies have reported diversity in the nutrient profiles of sugary food sources, and the fact that black flies sugar-feed (Brenner & Cupp, 1980; Hunter, 1977; Cupp & Collins, 1979; Davies & Peterson, 1956). However, there is little evidence of the diet selection pattern of black flies, and the factors that influence their sugar-feeding behaviour.

The present study tests two hypotheses. The first is that calorie content and nutritional composition of experimental sugary diets determine the diet selection pattern of female *S. venustum/verecundum* black flies. From this hypothesis, I predict that the flies will selectively feed on diets that contain the most calories per unit volume, and also contain the two amino acids in addition to the sugars. The second hypothesis is that the ecological fitness of female *Simulium* black flies feeding on experimental sugar diets is affected by the nutritional value of the diet. For practical reasons, nutritional value is defined as the total calorie content and the diversity of nutrients available in a unit quantity of diet. Survivorship, fecundity, and parasite infection intensity were used as indirect measures of ecological fitness.

Specific hypotheses and predictions are as follows:

Survivorship:

Hypothesis: Survival of female *S. venunstum/verecudum* is affected by differences in nutrient and calorie content of artificial diets.

Prediction: Black flies that feed on concentrated honeydew (the diet with highest calorie content) will live longer than others that feed on any other diet.

Fecundity:

Hypothesis: The fecundity of *S. rugglesi* black flies depends on the total calorie content of sugary diets they are fed with.

Predictions: Flies that feed on high calorie diets will have more eggs in their ovaries.

Parasite infection intensity (parasite load):

Hypothesis: Intensity of infection with midgut stages of *Leucocytozoon simondi* in *S. rugglesi* depends on the glucose concentration in diets.

Prediction: Flies that feed on the higher glucose diet, nectar will have fewer *L. simondi* parasites on their midgut walls.

S. venustum/verecundum was chosen for this study for two reasons: it is the most abundant species found at the Wildlife Research Station at the Algonquin Provincial Park (personal observation; Davies, 1952), and being heamatophagous, results from their study could be extrapolated to explain behaviors of other biting simullids. This study also used a *Leucocytozoon simondi-S. rugglesi-Anas bochas* model system to study the effect of sugar feeding on the level of parasite 'infection' in the simuliid vector. This is a natural model system that exists at the research site and has been used successfully by

many investigators for various studies (Bennett, 1960; 1963; Roller & Desser, 1973; Hazzard, 2003; Smith & Friend, 1982).

METHODS

Diet selection

Fifteen pairs of diet combinations were made with the six treatment diets. A diet in each pair was randomly assigned one of two colours. One of the diets in each pair was put in one corner of a screen-covered 30 cm X 17 cm X 10 cm plastic basket (feeding arena), and the other in the corner diagonal to it. This order of the diets in the feeding arena was reversed to serve as the counterbalance of the original diet pair, in a second set up, so that each diet pair had a counterbalance. This was done to control for possible non-uniformity in the intensity of ambient light around the arena. Each diet was colour coded (see control experiment below).

Groups of newly emerged female flies (~40) were randomly sampled into the feeding arenas and allowed to feed from the diet pairs for 24 hours. Black flies store sugars they feed on in their crop, which is located on the ventral side of their abdomen. The crops typically become greatly enlarged when a fly sugar-feeds and it is possible to see the color of the diet they have fed on through the translucent abdomen (Fig. 3.1). A water-soaked cotton ball was placed on top of the feeding arena to provide drinking water for the black flies. After the 24-hour period, the feeding arenas were put in a freezer briefly, to immobilize the flies. The ventral side of each individual's abdomen was inspected under a light microscope and the colour (indicating the kind of diet fed on) was recorded. The frequency of mixed feedings or flies that fed on both test diets was very low and as such excluded from analysis.

The proportion of flies that selected a diet in a pair was compared to the proportion of flies that selected that same diet in the counterbalance trial by use of the chi-squared test of independence at $\alpha = 0.05$. The outcomes of a trial and its counterbalance were combined if the chi-squared test of independence showed no significant difference between the two proportions. However, where the chi-squared test of independence showed significant difference in the outcomes, the proportions were not combined, and the data excluded from subsequent analysis. To assess the diet selection pattern of flies for a given diet pair, goodness-of-fit chi-squared tests were conducted on the combined data at $\alpha = 0.05$.

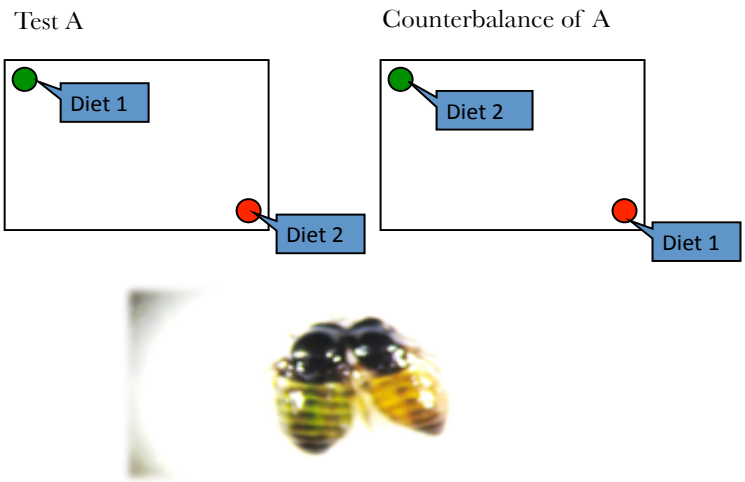


Fig. 3.1. A schematic of the placement of treatment diets. Also shown are black flies that had fed on coloured diets.

Controlling for potential confounders

To circumvent a potential confounding factor of differential attractiveness of dye colours to black flies, the most appropriate colours to use in the diet selection experiments were determined in preliminary choice experiments. According to Browne and Bennett (1980) blue is the most attractive colour to *S. venustum* while other colours available for use in the study viz. red, yellow and green, are not significantly attractive to the black flies. 10% sucrose was dyed red, green, yellow or blue with food dye (Club House™ food colour preparation) and presented in a feeding arena as described above. Two replicates of about 20 flies each were run for each colour combination. The number of individuals that had fed on each treatment (combination) was pooled and analyzed via chi-squared goodness of fit test. The colour combinations that showed no significant difference in their attractiveness was selected and used in the diet selection experiments.

To eliminate another potential confounding factor: the possible different levels of gustatory response of black flies to the individual nutrient components of the diets, control experiments were run with 5% (w/v) solutions of the sugars: sucrose, fructose, glucose, and melezitose, and 1% (w/v) solutions of the amino acids: asparagine and glutamine. Groups of *S. venustum/verecundum* females ($n \geq 30$) of the same age were provided one of the solutions by means of a dental wick soaked in the respective solution and distilled water by a similar means. Each solution was dyed blue. The flies were allowed to feed for 24 hours and then aspirated into 95% ethanol. The number of individuals having blue colouration in their abdomen (indicating a successful feeding on the solution) was counted and recorded as an indicator of the ability of component to

elicit a feeding response. The proportion of flies that fed on a solution was compared to the proportion that did not feed via chi-square goodness of fit test at $\alpha = 0.05$.

Effect of sugary diet on black fly survivorship

Groups of newly emerged *S. venustum/verecundum* black flies were randomly assigned to one of the treatment diets or distilled water in individual housing chambers and kept in an incubator. Each housing chamber was inspected every 6 hours until the individual in it died. During each inspection time, dead individuals were removed and diets replenished for survivors. Wing length of each carcass was measured as an estimator of body size, and used to assess whether body size significantly influenced survivorship. Survivorship was assessed as the proportion of test subjects alive at specific times during experimental trials. Estimates of survival distributions were done using the Kaplan-Meier plots. Assessment of overall group (diet treatment) differences in the survival distributions was done using the log-rank test. Follow-up testing between pairs of groups was done in conjunction with Bonferroni-corrected significance levels. Effects of diet and wing length on survivorship were assessed via ANCOVA at $\alpha = 0.05$.

Effect of sugary diet on fecundity

S. rugglesi collected from the sentinel duck hosts (see general methods) were randomly sampled into individual housing chambers and provided either nectar (N) or honeydew (H) diets in addition to distilled water in each case. Eight days after blood feeding, the flies were immobilized by brief chilling. The ovaries of each individual were dissected in a drop of phosphate buffered saline (PBS) (0.01 M, pH = 7.3) on a clean microscope slide. All eggs (irrespective of developmental stage), contained in the dissected ovaries were counted with the aid of a light microscope. The number of eggs per individual per treatment was recorded to represent the fecundity of the fly. Differences in the number of eggs per diet treatment were assessed by independent t-test at $\alpha = 0.05$.

Effect of sugary diet on parasite load

All blood-fed *S. rugglesi* collected from the sentinel duck hosts (see general methods), were randomly sampled into individual housing chambers and provided either concentrated nectar (CN) or concentrated honeydew (CH) diets. Eight days post infective blood feed, individuals were removed from the containers in which they were kept and immobilized by brief chilling on ice. Immobilized blackflies were quickly transferred to a drop of PBS (0.01 M, pH = 7.3) on a clean microscope slide and their midguts dissected. The dissected midguts were covered with a cover slip and inspected under a light microscope for the midgut stages of the *L. simondi* parasite, (oocysts). The number of oocysts per midgut per diet treatment was recorded as a measure of parasite load in the black fly. Differences in the number of oocysts per diet treatment were assessed independently by t-test at $\alpha = 0.05$.

RESULTS

Diet selection

Although there were no significant differences in the attractiveness of any colour in all the colour combinations, the red and green dye combination was chosen for the experiments because they showed the most contrast when being compared under a light microscope. Neither red nor green food dye colour used to dye treatment diets influenced the diet selection of the female *S. venustum/verecundum* used in the study (Fig. 3.2; χ^2 (1, 31) = 0.03, p = .86).

To control for the possibility of differential levels of gustatory response of the flies to dietary components, a feeding response test was conducted. In all treatments, the proportion of female *S. venustum/verecundum* flies that fed on a nutrient solution was significantly greater than those that fed on the alternative 'diet', distilled water (Fig. 3.3; χ^2 goodness-of-fit test; $p < .001$ for each). A chi-squared test of homogeneity via contingency tables was conducted to compare the proportion of flies that fed on nutrient solutions among the treatments.

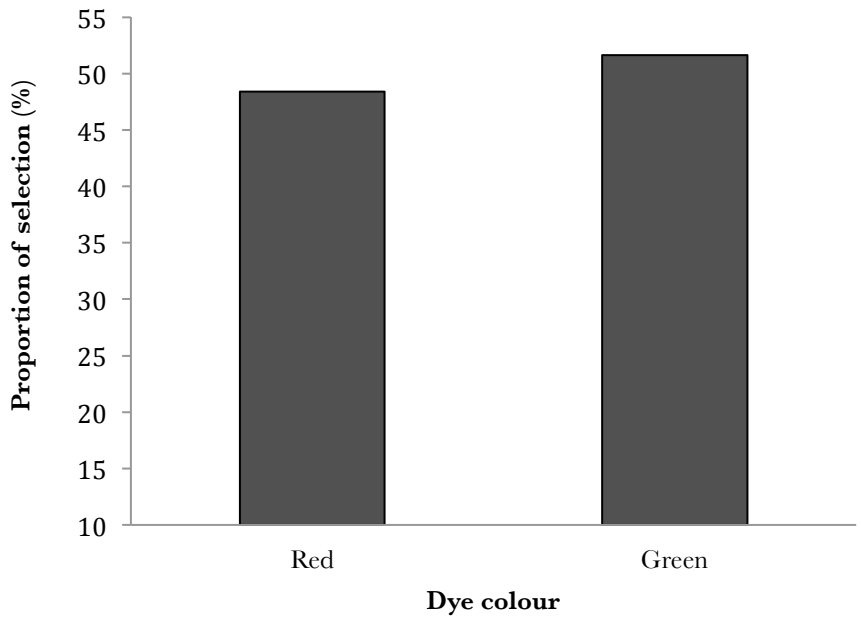


Fig. 3.2. Effect of dye colours on selection pattern of black flies: neither red nor green food dye was significantly more attractive to female *S. venustum/verecundum* black flies (χ^2 goodness-of-fit test, $p = .86$). Attractiveness was assessed as the proportion of flies that fed on a diet dyed with a particular food colour.

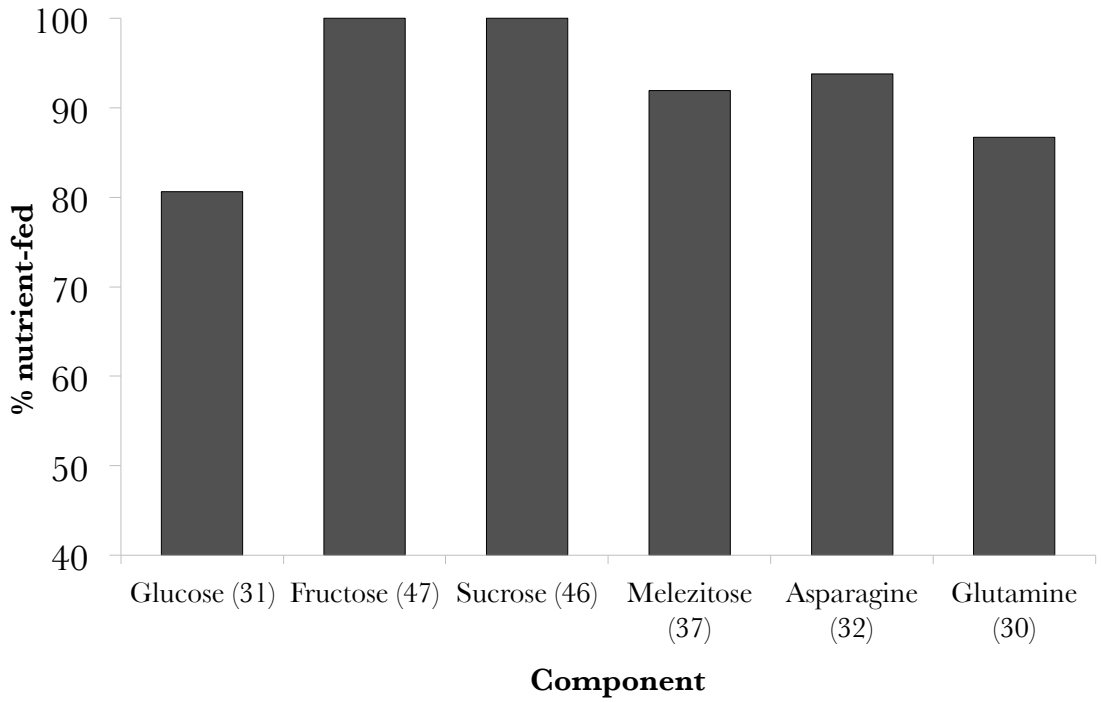


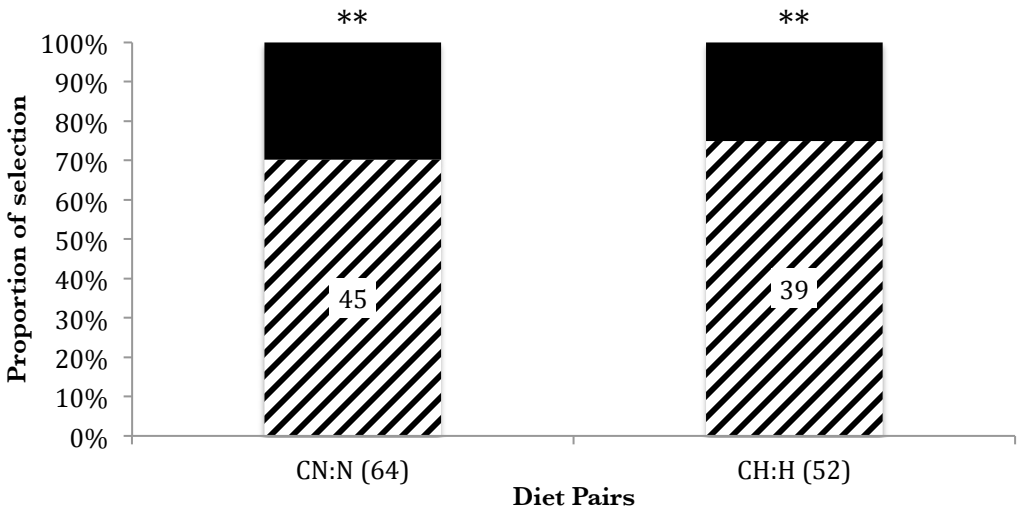
Fig. 3.3. Proportion of female *S. venustum/verecundum* black flies that fed on nutrient solutions as opposed to distilled water over a 24-hour period. Sugars were 5% w/v and amino acids 1% w/v. There was no significant difference between treatments after 5000 Monte Carlo simulations of the proportions and pair-wise comparison using the Marascuilo procedure. In all treatments, significantly more flies ($p < .001$ for each) fed on the nutrient solution than they did the distilled water. Numbers in brackets = N.

The effect of total calories per mL of diet, amino acid content, and melezitose content of diets on the foraging behaviour of female *S. venustum/verecundum* black flies was assessed as described earlier. It was observed that the factors listed here either by themselves or in combination with another, influenced the foraging decisions of the black flies studied. The results of these experiments are presented below under the headings of the factors hypothesized (*post hoc*) to be influencing the foraging pattern of the flies. In all 15 pairs of observations were made; each pair made up a test and its counterbalance. Only 2 of the 15 pairs of observations failed the independence test via chi-squared; this could have been due to experimental error or some other prevailing ambient factor when these particular experiments were ran. Eight of the remaining 13 yielded significant results.

Calorie effect

The proportion of flies that fed on the higher calorie diet was higher than those that fed on the lower calorie diets. Approximately 70% of all flies selected concentrated nectar (higher calorie) over regular nectar (lower calorie). This was statistically significant (Fig. 3.4 (a); χ^2 goodness-of-fit (1, 64) = 10.56, $p < .01$). Significantly, more flies selectively fed on the higher calorie concentrated honeydew than did the lower calorie regular honeydew (Fig. 3.4 (a); χ^2 goodness-of-fit (1, 52) = 13.00, $p < .01$). This represented 75% of all selections. Even with the addition of amino acids, the diet selection pattern of the flies did not change. They significantly fed more on the higher calorie nectar diet than the lower calorie one although it contained the amino acids (Fig. 3.4 (b); χ^2 goodness-of-fit (1, 41) =12.90, $p < .001$). Similarly, significantly more flies fed on the higher calorie compared to honeydew diet (Fig. 3.4 (b); χ^2 goodness-of-fit (1, 56) = 18.29, $p < .001$).

(a).



(b).

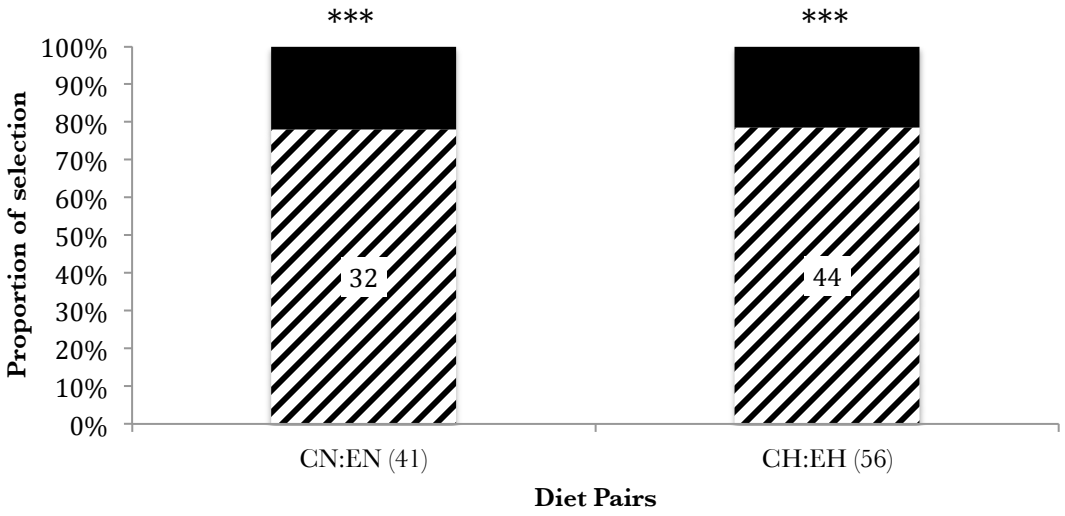


Fig. 3.4. Proportion of female *S. venustum/verecundum* black flies that fed on diets in paired choice experiments. (a) The higher calorie diets (hatched) were selected more often in the nectar (first column) and honeydew (second column) preparations. (b) Higher calorie diets (hatched) were selected more frequently than the lower calorie amino acid-containing diets (solid) in both the nectar (first column) and honeydew (second column) preparations. Figures in brackets = N; ** $p < .01$; *** $p < .001$. N=nectar; H=Honeydew; CN= concentrated Nectar; EN=enriched nectar; CH=concentrated honeydew; EH=enriched honeydew.

Amino acid effect

Significantly more black flies fed on the honeydew that had been enriched with asparagine and glutamine than those that fed on the non-enriched diet (Fig. 3.5; χ^2 goodness-of-fit (1, 47) = 11.26, $p < .001$). The selection represented approximately 74% of a total of 47 selections. The nectar and enriched nectar diets comparison failed the chi-squared test of independence on the outcomes of the test and its counterbalance. The result of that test was not included in the data analyses and thus is not shown.

Combined effects

The effect of calorie content, melezitose content and amino acid content of diets on the foraging decisions of the flies was in some instances impossible to isolate due to the composition of the diets. In such case, at least two of the factors seem to exert a combined influence on the diet selection patterns of the black flies. Generally, the flies appeared to selectively feed on the calorie-rich, melezitose-containing diets. This selection pattern was significant only when amino acids were present in at least one of the diets to be selected from (Fig. 3.6 (a); χ^2 goodness-of-fit (1, 48) = 16.33, $p < .001$, for honeydew versus enriched nectar comparison; χ^2 goodness-of-fit (1, 55) = 8.02, $p < .01$, for enriched honeydew versus enriched nectar comparison, and χ^2 goodness-of-fit (1, 77) = 45.21, $p < .001$, for concentrated honeydew versus enriched nectar comparison). In one instance, the enriched honeydew versus nectar comparison, where all three factors could influence diet selection, no significant selection for any diet was observed (Fig. 3.6 (b); χ^2 goodness-of-fit (1, 43) = 1.14, $p = .29$).

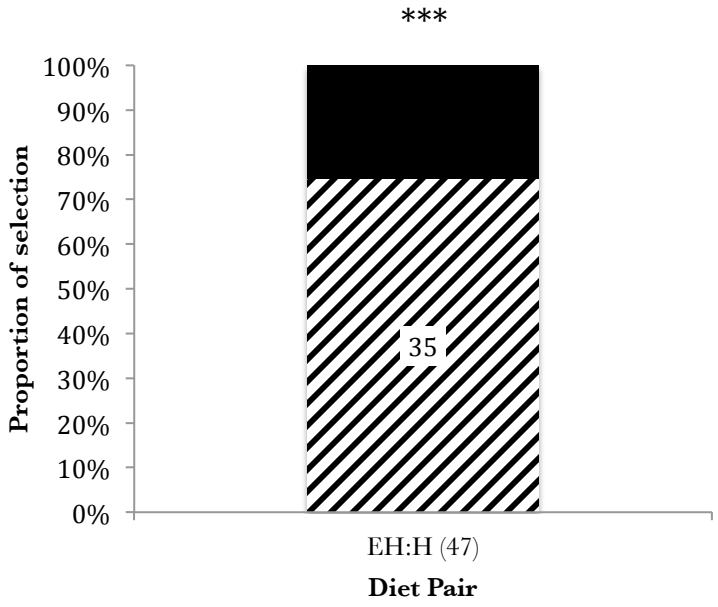
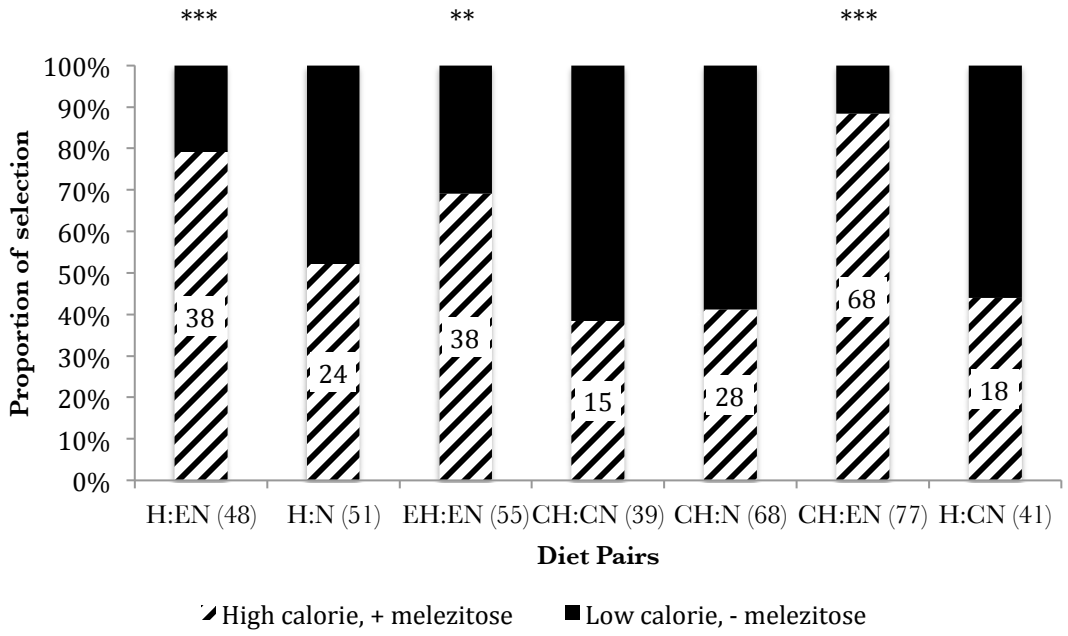


Fig. 3.5. Effect of amino acids on diet selection pattern of female *S. venustum/verecundum* black flies. EH=enriched honeydew H=honeydew. Significantly more flies fed on the enriched diet (hatched) than the regular diet (solid). *** $p < .001$. N = 47.

(a)



(b)

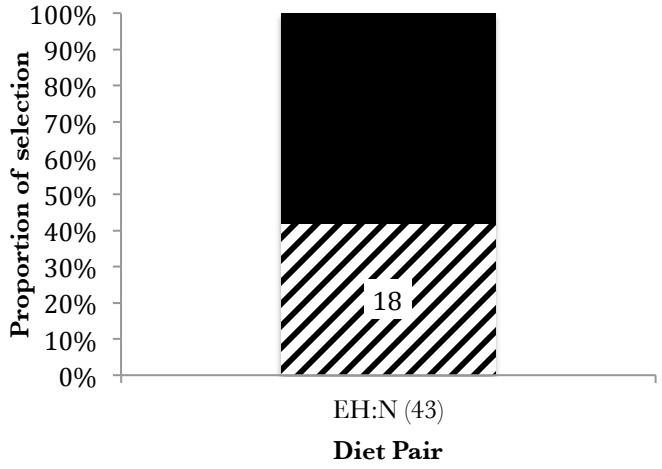
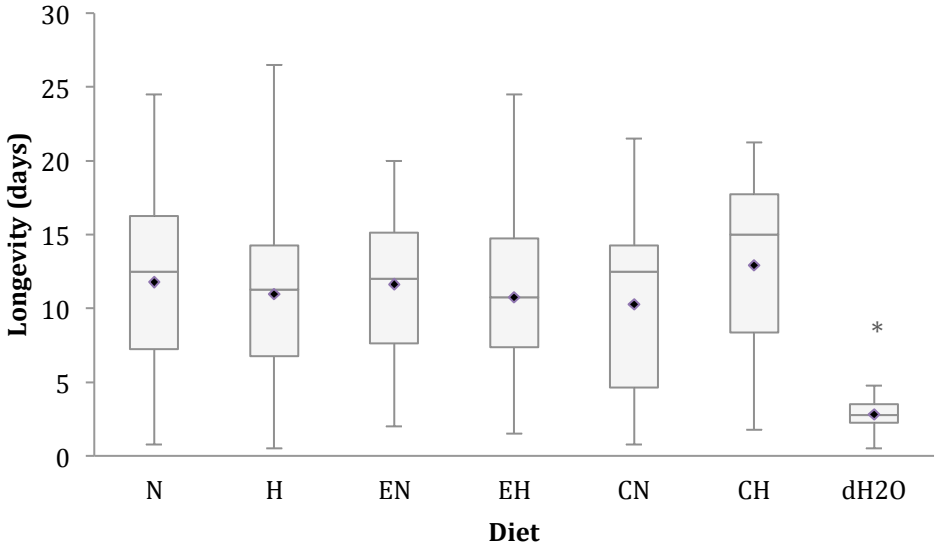


Fig. 3.6. Combined effects of diet factors on the diet selection patterns of female *S. venustum/verecundum* black flies. (a) The flies seemed to discriminately feed on the calorie-rich, melezitose-containing diets. (b) The combined effect of all diet factors did not influence the diet selection pattern of the black flies $p = .29$. H=honeydew, N=nectar, EH=enriched honeydew, EN=enriched nectar, CH=concentrated honeydew, CN=concentrated nectar. Figures in brackets=N, ** $p < .01$, *** $p < .001$. Bottom stack represents first diet in the diet-pair label.

Effect of sugary diet on black fly survivorship

Black flies that fed on concentrated honeydew, CH lived longer than those that fed on any other diet (M = 12.92, SEM = 0.86) and 3 days longer than the overall average of approximately 10 days. Flies reared on enriched honeydew, EH lived the shortest of all the treatment groups (M = 10.27, SEM = 0.84). A log-rank test analysis of the survival distributions of the flies per treatment revealed a significant effect of treatment on survivorship Log-rank (6, 351) = 222.41, $p < .0001$ (Fig. 3.7 (b)). Follow up pair-wise comparison via Tukey (HSD) test revealed no significant difference between the diet treatments (Fig. 3.7 (a) at the Bonferroni-corrected significance level of 0.002) but significant differences between the survivorship of flies on the diets and those fed the control (distilled water). An analysis of covariance [between-subjects factor: diet; covariate: wing length] revealed a main effect of diet, F (6, 351) = 20.97, $p < .0001$) but no effect of wing length F (1, 351) = 2.70, $p = .01$ on survivorship. These results suggest that no single diet significantly improved the survivorship of any group of flies over another.

(a)



(b)

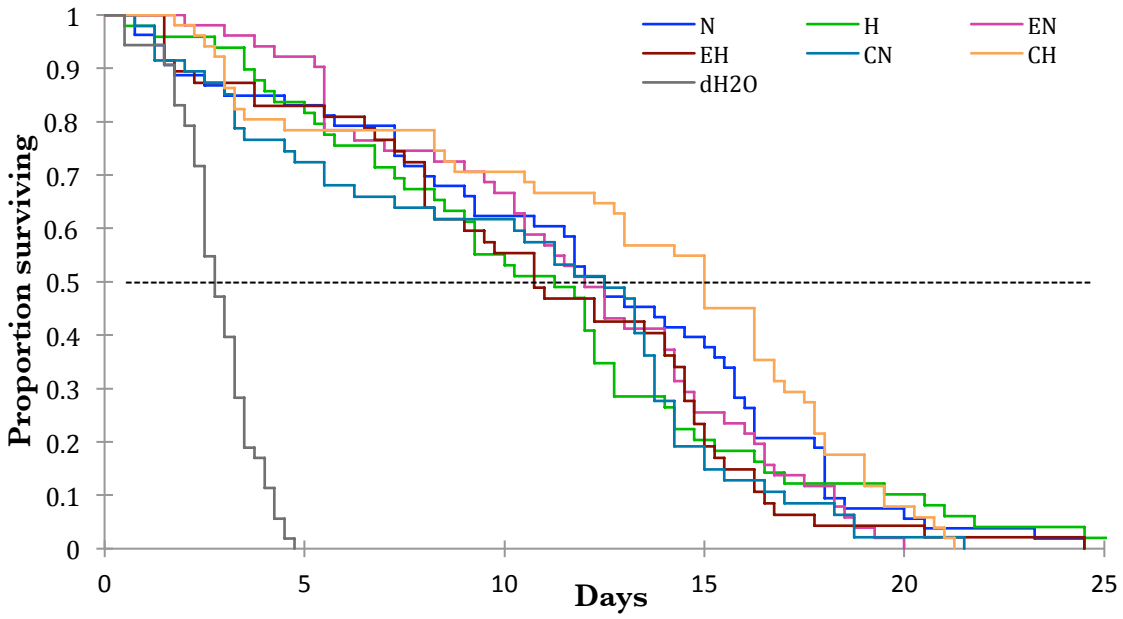


Fig. 3.7. Effect of sugar feeding on survivorship of female *S. venustum/verecundum* black flies (a) descriptive statistics on the survivorship of flies per treatment (b) a Kaplan-Meier plot showing the survival distributions of the flies per treatment and a 50% survivorship line.

Effect of sugary diet on fecundity

An unpaired t-test analysis was conducted on the number of eggs counted for individuals fed on either nectar (N) or honeydew (H). There was no significant difference in the egg counts for N (M = 112.97, SEM = 10.59) and H (M = 108.36, SEM = 11.24); t (41) = 0.30, p = .77 (Fig. 3.8); implying that sugary diets have no effect on the fecundity of *S. rugglesi* black flies.

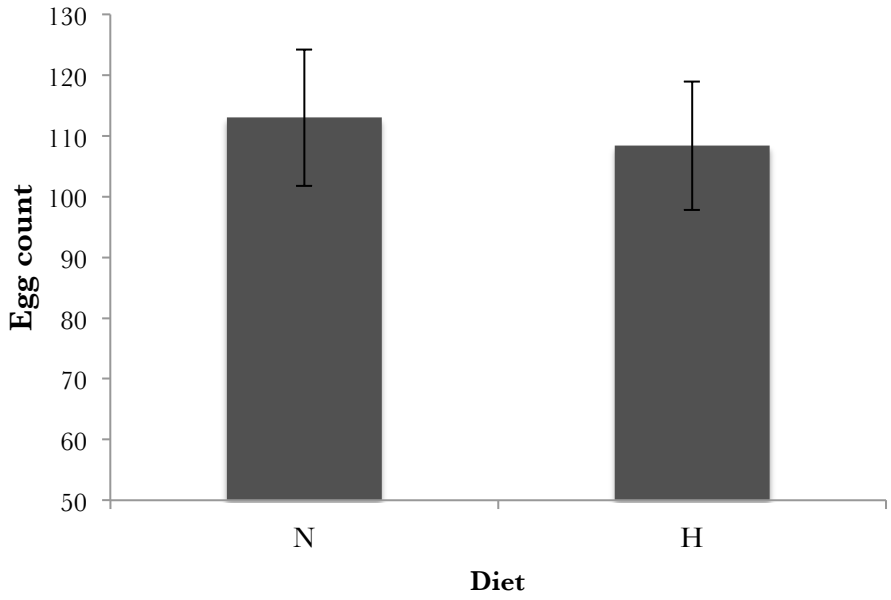


Fig. 3.8. Influence of sugary diet on the mean number of eggs present in the ovaries of *S. rugglesi* black flies 8 days after blood feeding. Diet did not affect mean egg count (fecundity). H=honeydew, N=Nectar, N = 21 for each treatment, error bars = standard error of the mean.

Effect of sugary diet on parasite load

This experiment investigated the influence of sugar diet on the mean number of oocysts found on the midgut wall of black flies. An independent t-test was conducted to compare the oocyst counts of the flies fed on concentrated nectar CN and those fed on concentrated honeydew, CH. There was a significant difference in the oocyst count for CN ($M = 150.6$, $CI = 26.3$) and CH ($M = 95.3$, $CI = 17.3$); $t\ (56) = 3.53$, $p < .01$ (Fig. 3.9). The results suggest an influence of sugar feeding on the intensity of parasite infection in a black fly.

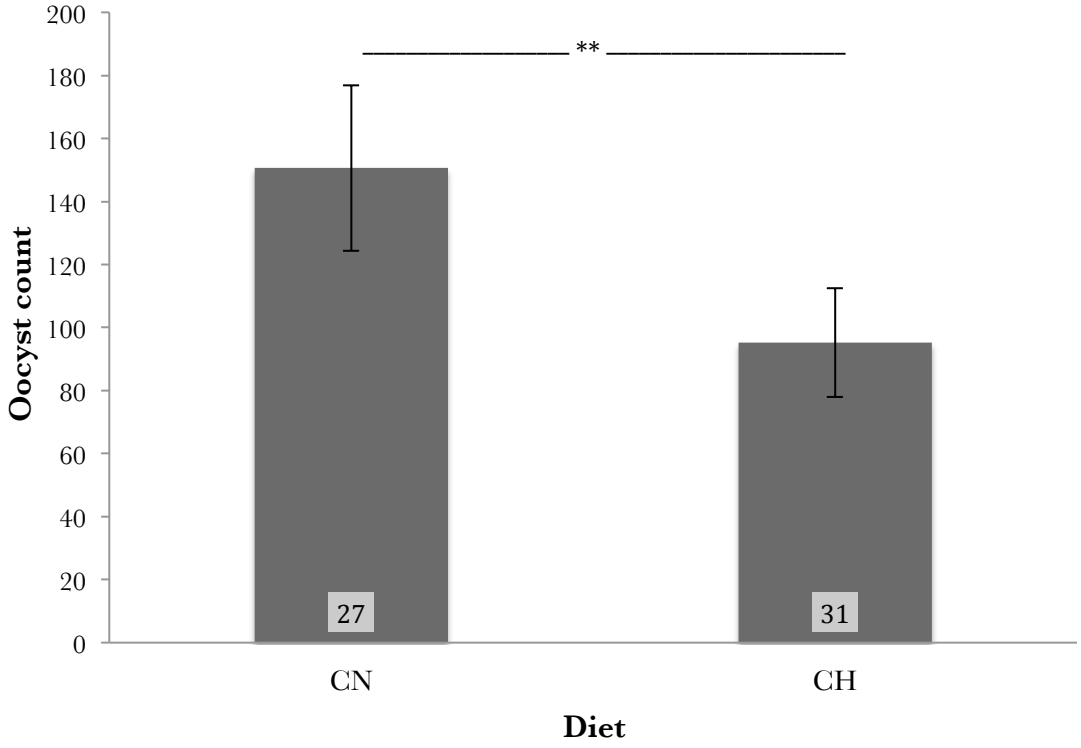


Fig. 3.9. Influence of sugary diet on the mean number of *L. simondi* oocysts on the midgut walls of *S. rugglesi* black flies. Flies that fed CN (the diet with the higher glucose content) showed significantly higher levels of parasite load. CN=concentrated nectar, CH=concentrated honeydew, numbers at base of column = N, error bars = 95% confidence interval, ** $p < .01$.

DISCUSSION

The present study provides information useful in the advancing of current knowledge and understanding of the sugar foraging behaviour of female heamatophagous black flies. In preliminary tests to assess the suitability of colour combinations for dying treatment diets, neither red nor green food dye colour influenced the selection of the female *S. venustum/verecundum* (Fig. 3.2). This observation compares favourably with the findings of Browne and Bennett (1980) who reported blue to be the most attractive colour to host seeking females but not the ability of red and green among other colours. This suggests that, red and green food dyes can be adopted as standards dyes in diet selection experiments involving these black flies.

The absence of significant differences in the gustatory response of female *S. venustum/verecundum* to the nutrient components of the experimental diets shows that diet-specific differences in ability to elicit taste and feeding response cannot account for observed difference in the feeding behaviour of the flies (Fig. 3.3). Combinations of the dietary nutrients may however produce a different gustatory response from the flies and possibly explain any observed differences in diet selection pattern. Presently, there are no studies on the phagostimulatory response of black fly labella and tarsal hairs to sugars and amino acids. Dethier (1955) notes that there are at least two modalities of chemo-sensation in flies: acceptable and unacceptable, and that flies can readily distinguish between water and sucrose solutions as dilute as 1×10^{-7} M. Shiraishi & Kuwabara, (1970) demonstrated that L-glutamate and L-aspartate inhibit the response of sugar and water receptors on the labella sensory hairs of fleshfly, *Boettcherisca peregrina,* and the blowfly, *Phormia regina*. Further, Goldrich (1973) reported that the black blowfly

Phormia regina was unable to distinguish between these same amino acids in electrophysiological studies of the labella sensory hairs. Taken together, these results and those of the present study suggest that although there is no difference in the ability of individual nutrients to produce feeding responses from the flies, when combined together, these nutrients are able to elicit strong feeding responses.

The mechanisms that underlie the ability of female *S. venustum/verecundum to* selectively feed from sugary food sources is not known. In nature, nectars and honeydews used by these flies have dietary components that occur in various combinations and have diverse distributions (Burgin, 1996) as such, as predicted by MacArthur and Pianka (1966), foraging black flies should feed from those diets that offer the most net energy gain i.e., flies should forage optimally, selectively foraging on diets with enough caloric content to offset the energy invested in foraging for them. In the present study, female *S. venustum/verecundum* appeared to be able to discriminate between sugary food sources since 8 of 13 paired choice experiments produced significant differences in the diet selection patterns between pairs.

Calorie content of diets played a role in the foraging behaviour of the flies. It is likely that the flies are selecting the higher calorie content diet principally because in all cases they were the more concentrated diet. Thus the selection pattern of flies is perhaps better explained as a response to the concentration (%w/v) of the diets. Some dipterans process sugary food differently based on the total solute concentration. In black flies and mosquitoes, ingested sugary solutions are temporarily stored in the crop and passed on in small amounts to the midgut for digestion. Friend (1978) reported that the transitional time of sugar solution in the crop of the mosquito *Culiseta inornata* depends on the

concentration of the solution; dilute solutions having a more rapid transit time than concentrated solution.

For a heamatophagous species such as *S. venustum/verecundum,* feeding from a high calorie, more concentrated diet has obvious benefits. An individual saves time and abdominal space when it feeds on such diets. The individual does not need to forage for a long time to meet caloric requirements because concentrated diets tend to have more calories per unit volume. Since the crop (which stores sugary foods) and the midgut (which stores blood) physically occupy the same abdominal cavity, there is "physical tradeoff for abdominal space between blood in the midgut and sugar in the crop" (Ma, 2010); i.e., a replete sugar meal eliminates the possibility of a replete blood meal, taking a small amount of high calorie diet allows the fly to meet the energetic budget for blood seeking and have the room in the abdominal cavity to accommodate a fully distended midgut upon blood feeding. Thus female *S. venustum/verecundum* selectively feeding on concentrated diets (Fig. 3.4) does not only obtain an immediate benefit of high calorie intake per feeding time, but also potential reproductive fitness benefit since blood feeding is important in the development and maturation of eggs in this species.

There were a few cases of mixed feeding, where flies initially fed on a diet and then switched to another one. This observation was possible because the coloured diets were easily visible in the crop through the translucent abdomen of the flies. In all cases (results not shown), the flies initially fed on the lower calorie diet in the pair and then switched to the higher calorie diet in the pair.

Where a difference in caloric content (or concentration) was not a factor in diet selection, the flies appeared to selectively feed from the amino acid-containing diet (at least in the honeydew diets) (Fig. 3.5). The nectar and enriched nectar diets comparison

failed the chi-squared test of independence on the outcomes of the test and its counterbalance. This could have been due to experimental error and a repeat experiment would be necessary to allow proper interpretation of the results. Since amino acids (whether essential or not) are the biosynthetic precursors of many biomolecules including proteins and nucleotides, a diet containing amino acids may be selected over one that does not. Dadd (1973) considers glutamine and asparagine nonessential to insects but states that their inclusion in diets as supplements may enhance growth of some insects as it does in the blowfly, *Phormia regina*.

As previously stated, in some instances, explanation of observed diet selection pattern could not be done without considering the combined effect of some of the diet-specific factors. In many instances the combined effects of calorie and melezitose content seemed to influence the foraging behaviour of the flies. Female *S. venustum/verecundum* black flies appeared to selectively feed from those diets that were more concentrated (thus had higher calories) and also contained melezitose than those without melezitose and were lower in calorie content (Fig. 3.6). It is interesting to note that this selection pattern was only significant in the situations where one of the diets in a pair contained amino acids. In those situations, the black flies selectively fed on the higher calorie diets that contained melezitose but no amino acids. It is impossible to know for certain whether the flies were avoiding the amino acids or selectively feeding on the melezitose-containing diets. Taken together, the flies generally selected higher calorie diets over lower ones irrespective of whether they contained melezitose or amino acids except when there were no differences in calorie content between the diets.

Effect of sugary diet on black fly survivorship

Sugars have been established to be essential to black fly survival. In this study, flies maintained on water lived an average of 2.8 days. This observation compares fairly well with other results previously reported. Stanfield and Hunter (2010) found that *S. venustum/verecundum* species complex fed only water lived to about 4 days. Rodriquez-Perez et al, (1995) reported that sugar-starved simuliids lived only 2 days. Davies (1952) also reported that female *S. venustum* black flies provided with distilled water lived shorter than those that were provided with varying concentrations of sucrose and 50% of individuals died approximately 1.8 days after the start of the experiments. The results in the present study support previous claims of sugar feeding being essential to black fly survival. In the present study, no diet significantly improved the survivorship of any group of flies over another (Fig. 3.7). Implying that whereas sugar feeding is essential for black fly survival, sugar diet properties such as caloric content, amino acid content and melezitose content do not enhance the survivorship of flies.

Effect of sugary diet on fecundity

There was no significant difference in the fecundity of flies fed on honeydew, H and those fed on nectar, N (Fig. 3.8). This implies that sugar diet does not affect fecundity in *S. rugglesi* black flies. This finding is similar to that reported by Hazzard (2003). Davies & Peterson (1956) found average of 256 eggs in *S. rugglesi,* which is markedly different from the average of about 110 eggs obtained for both fly groups together. This lack of compatibility between the two observations can be explained by the fact that the current study manipulated the nutritional intake of the flies after blood

feeding and then assessed fecundity whereas the previous work assessed fecundity in the wild-caught flies without interfering with their diet. The flies in Davies & Peterson (1956) work may have had access to various plant-derived macronutrients such as proteins from pollen-contaminated nectars thus increasing their intake of proteins (essential nutrient in egg maturation) more than the lab-reared flies in the present study.

Effect of sugary diet on parasite load

The significantly lower levels of parasite load in the group of flies fed with the honeydew diet suggest that sugar feeding has an effect on the parasite levels that a black fly can support (Fig. 3.9). This observation is in line with results obtained by Hunter and Recce (unpublished data) when they provided groups of *Anopheles stephensi* with similar diets. In that study, the investigators found that the group of mosquitoes that fed on the honeydew diet had fewer oocysts stages of the *Plasmodium berghei* parasite on their midgut wall. Given that the glucose content in the CN diet is about 2.3 times more than that contained in the CH diets, per unit, the higher glucose content probably allowed the nectar-fed group of flies to support the development of more numbers of the parasite. Glucose may be essential to the development of *L. simondi*; a lack of which probably led to relatively reduced levels of parasite load in the honeydew-fed black flies. Rivero & Fergusson (2003) reported an increase in glucose consumption in *Plasmodium*-infected mosquitoes and suggested a parasite-mediated change in vector sugar-foraging behaviour. In other words, parasite-infected mosquitoes fed more on glucose to replenish the resources depleted by the *Plasmodium* parasite. Glucose may influence the ability of *S. rugglesi* to efficiently vector *L. simondi*.

The higher caloric content of the honeydew diet, CH probably improved the energetic budget of the black flies and allowed them to invest in physiological responses to rid themselves off the *Leucocytozoon* parasite. The CH diet contained about 1.7 times more kilocalories per milliliter of diet than the CN diet. It is reasonable to expect that immune response to parasite infection would incur an energetic cost. Given that the treatment diets were the only source of energy available to the flies during the study, and the fact that other metabolic activities had to be maintained, it is suggested that difference in caloric content in foods would improve the ability of flies to mount more elaborate and efficient forms on immune response to the parasites.

CHAPTER 4:

INFLUENCE OF SUGARY DIETS ON LIFE HISTORY-RELATED TRAITS OF MOSQUITOES

INTRODUCTION

Mosquitoes are generally best known for the blood feeding behaviour of some females. The blood feeding behaviour makes them vectors of numerous arboviruses and other parasitic disease causing pathogens affecting human and animal health (Jones et al., 2004). Whereas blood feeding is necessary in some species for ovarian development, sugar feeding provides males and females with energy for flight and the maintenance of metabolic activities. Since male mosquitoes do not blood feed their only source of caloric intake as adults is by sugar feeding; in fact sugar feeding has a direct impact on the reproductive fitness of males because it provides energy for swarming and mating finding. Females also depend on sugars for energy mainly for flight to increase their chances of being inseminated and finding blood (Gary & Foster, 2004).

Sugar feeding is the principal source of energy for mosquitoes (Foster, 1995). Even the African malaria vector, *Anopheles gambiae,* which had previously been thought of as not sugar feeding at all, has been shown to sugar feed (Gary & Foster, 2004). In fact female *An. gambiae* take sugar meals from peridomestic plant and honeydew sources before seeking blood (Gary & Foster, 2004). Manda et al. (2007) indicated that *An. gambiae* obtained sugars primarily from flowers occasionally feeding from exudates from stem and leaves. Mosquitoes utilize sugars from varied sources including: homopteran honeydews, floral and extra-floral nectars, exudates from plant parts, fungal excretions, rotten fruits, etc. (Foster, 1995; Manda et al., 2003; 2007). These sugary food sources

usually exist as solutions but may crystallize in low humidity environments. Mosquitoes have been reported as being able to feed on crystalline sugars (Clements, 1992).

Gary and Foster (2004) found no evidence of mosquitoes penetrating plant tissue with their proboscis to feed on plant-derived sugars. Instead the mosquitoes studied probed around the plant tissue and readily aggregated on extra-floral nectars or phloem sap liberated from cut ends of the plant. Mosquitoes can sugar feed for as long as 30 minutes (Clements, 1992). Imbibed sugar solutions are stored in the crop and released in small amounts into the midgut, where digestion takes place (Friend, 1978). The transit time of sugary foods through the crop depends on its total solute concentration; dilute solutions transit faster than more concentrated solutions. Mosquitoes are equipped with digestive enzymes, which enable them to digest the oligosaccharides found in the common sugar food sources. Wilkins and Billingsley (2001) found glucosidases, galactosidases, aminopeptidases and glutamyl-transferases associated with midgut microvilli of *An. stephensi*.

Many dipterans possess receptors on their tarsal hairs to allow chemosensory perception and response to glucose, fructose, and sucrose in sugar solutions (Wieczorek & Wolff, 1989). In a review, Friend (1978), reported that mosquitoes possess chemosensory tarsal and antennal hairs, which respond to water, sucrose, maltose, and glucose when stimulated; consequently, the proboscis is deployed to initiate ingestion of the sugar solution. *An. gambiae* are capable of discriminating between sugary food sources from different plants and even floral and extra-floral nectars from the same plant (Manda et al., 2007). The sugar content, accessibility of sugar solution to foraging mosquitoes and the inclusion of other non-sugar nutrients such as amino acids may influence the foraging pattern of mosquitoes.

Mosquitoes appear to display a preference for sugars produced by certain plant parts. Manda et al. (2007) reported that *An. gambiae* preferred to feed from plant parts that produced extra-floral nectars with high concentrations of sucrose, fructose and glucose. The study also reported that the oviposition success of the mosquitoes studied was highest when they were fed with sugary foods from the *P. hysterophorus* plant and suggested that there may be other factors apart from total sugar concentration that influence fitness and thus affect the preference of mosquitoes for particular food sources. These other factors could be amino acids. The investigators also found that despite the high sugar content of the floral nectar of *Latana camara*, *An. gambiae* did not feed on it due to the rather long corollas of the flowers, which made the nectar physically inaccessible to foraging mosquitoes.

Sugar feeding has been found to influence mosquito survivorship both in laboratory and semi-field experiments. Both extra-floral nectar and mealybug honeydew extended the survivorship of mosquitoes that fed on them above that of those flies that were provided with water only; the effect of these diets on survivorship was similar to that of 50% sucrose solution (Gary & Foster, 2004). Survivorship of female *An. gambiae* was influenced by the amount of fructose they imbibed when provided various sugary meals; the higher the amount of fructose ingested, the higher the survival of the mosquitoes (Gary & Foster, 2004). Briegel (2003) found that the higher the concentration of sucrose solution on which female *An. gambiae* fed, the longer they lived. Taken together, these results indicate that sugar feeding is essential to the survival of mosquitoes and it extends their survivorship and that fructose and sucrose contents of sugary food sources are important factors that influence survivorship.

Ingestion of sugary diets may not be always beneficial to a female *Anopheles*

mosquito. In addition to limiting the total volume of blood that can be ingested, through reduction of the shared abdominal cavity, (Ma, 2010), feeding on sugary diets potentially can reduce the tendency of female mosquitoes to blood feed. Both nectars and honeydews contain the amino acid glutamine (Baker & Baker, 1977), which is converted to glutamate by glutaminase in dipteran tissue, which is further metabolized into γ-amino butyric acid (GABA) by the enzyme glutamate decarboxylase (Dowton & Kennedy, 1986; Richardson et al., 2010). GABA is known to have antifeedant properties in phytophagous insects (Bown et al., 2006; Gonzalez-Coloma et al., 2002; Shelp et al., 2006). *An. gambiae* possess the equivalent of the *Drosophila GAD1* (glutamate decarboxylase) gene, which has highly specific activity to glutamate (Richardson et al., 2010). It is reasonable to hypothesize that *An. stephensi* also possess this enzyme since according to Richardson et al. (2010), "counterparts of the *Drosophila GAD1* gene are found in all insect species whose genome are currently available". Thus feeding on sugary diets may limit feeding response of mosquitoes to blood.

Anopheles stephensi is the principal vector of malaria and dengue fever in India (Mandal et al., 2011; Sharma & Hamzakoya, 2001). It is capable of breeding in pools of water that collect in ditches, trash and even in chlorinated water storage containers around human dwellings (Mandal et al., 2011). Blood can be used as an energy source, but the fact that blood feeding is inherently risky due mainly to host defense mechanisms makes is a limiting resource thus very few species actually do this (Clements, 1992). Blood feeding provides the proteins needed for the development of ovaries in some species (Briegel, 2003). Blood feeding *Anopheles stephensi* can hold a maximum of 2 – 10 μL of blood in their midguts (Briegel & Rezzonico, 1985). After blood feeding, mosquitoes rest and begin digesting the blood during this time, they rarely forage for

sugar (Clements, 1992).

This chapter reports experiments to test two hypotheses. First, that the diet selection pattern of female *An. stephensi* mosquitoes is determined by the calorie and nutrient content of the available diets. Following this hypothesis, I predict that the mosquitoes will selectively feed on diets that contain the most calories per unit volume, and contain the two amino acids in addition to the sugars. Second, that survivorship and the amount of blood taken by female *An. stephensi* mosquitoes is determined by concentration and the total calories contained in sugary diets on which the feed. Based on this hypothesis, I predict that the mosquitoes that will feed on higher concentrated diet will live longer than others that feed on any other diet, and take less blood when they blood-feed.

METHODS

Diet selection

A two-sided choice experiment was done in pairs; a diet in each pair was randomly assigned one of two colours found in preliminary experiments not to influence the selection pattern of female *An. stephensi*. One of the diets in each pair was put on one side of the centerline on the base of an ice cream tub (feeding arena), and the other on the other side. This order of the diets in the feeding arena was reversed to serve as the counterbalance of the original diet pair, in a second set up, so that each diet pair had a counterbalance. This was done to control for possible non-uniformity in the intensity of ambient light around the feeding arena. A water-soaked cotton ball was placed on top of the feeding arena to provide drinking water for the mosquitoes.

Groups of approximately 30 newly emerged female mosquitoes were put into the feeding arenas and allowed to feed from the diet pairs available for 24-hours. Mosquitoes store sugars they feed on in their crop, which lies in the ventral side of their abdomen. The crops typically become greatly enlarged and it is possible to see the color of the diet they have fed on through the translucent abdomen. After the 24-hour period, the feeding arenas were put in at freezer briefly, to immobilize the mosquitoes. The ventral side of each individual's abdomen was inspected under a light microscope and the colour (indicating the kind of diet fed on) was recorded.

The proportion of flies that selected a diet in a pair was compared to the proportion of flies that selected that same diet in the counterbalance trial by use of the chi-squared test of independence at $\alpha = 0.05$. The outcomes of a trial and its counterbalance were combined if the chi-squared test of independence showed no significant difference between the two proportions. However, where the chi-squared test

of independence showed significant difference in the outcomes, the proportions were not combined, and discarded from subsequent analysis. To assess the diet selection pattern of flies for a given diet pair, goodness-of-fit chi-squared tests were conducted on the combined data at $\alpha = 0.05$.

Effect of diet on amount of blood meal taken

Groups of newly emerged female mosquitoes were sampled into containers and provided one of the following diets: nectar (N), honeydew (H), or 1% (w/v) asparagine, glutamine or arginine *ad lib*. Four days afterwards, they were allowed to blood feed from infected mice (with known infection intensity), and then housed in individually in housing chambers (Fig. 4.1). A housing chamber consisted of a scintillation vial containing a water-soaked dental wick in a small dram vial, which was fixed to the bottom of the scintillation vial by plasticine. The scintillation vial was covered with fine netting spread over a strip of filter paper and held in place with elastic bands. Each housing chamber also contained a thin strip of filter paper on which they could excrete the waste products of blood digestion.

Mosquitoes convert hemoglobin in blood meals to hematin, which is excreted as reddish-black matter, which can be used as an estimate of the size of the blood meal (Briegel, 2003). Three days after blood feeding, the quantity of blood taken by each individual was determined by spectrophotometry. The hematin pellets removed from the filter papers were dissolved in 1mL 1% Li_2CO_3. 100 µL aliquots of the resulting solution was then diluted in 1mL 1% Li_2CO_3 and scanned with a spectrophotometer to determine the absorbance at 387 nm (Briegel et al., 1978). The concentration of hematin excreted per fly per treatment was calculated from the formula: $c = A_{387} / \xi$, where c is the

concentration (M), A_{387} is the absorbance of the sample at wavelength of 387 nm and ξ is the molar extinction coefficient of hematin, 85, 000 $M^{-1}cm^{-1}$, (Fernandes & Briegel, 2005). An ANCOVA was performed to assess the effect of diet and infection level on the amount if blood taken. Pair wise differences in effect of amino acid on amount of blood taken was assessed by Tukey's HSD.

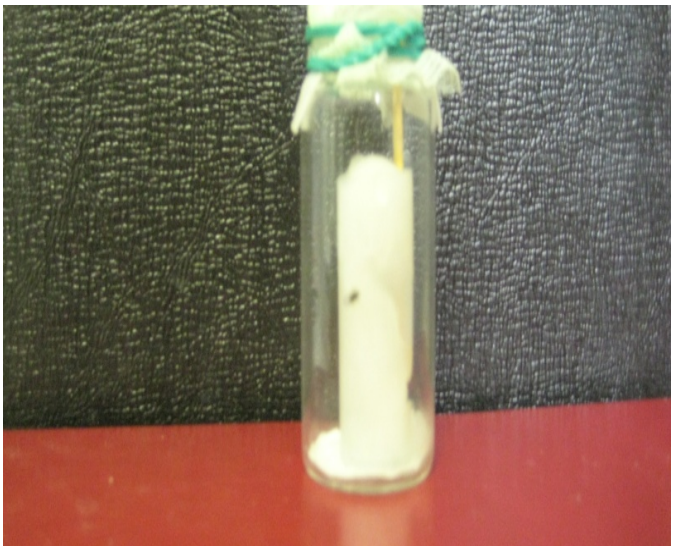


Fig. 4.1. Apparatus used to house experimental mosquitoes individually.

Effect on sugary diet feeding on mosquito survivorship

Groups of newly emerged *A. stephensi* mosquitoes were randomly assigned to one of the treatment diets or distilled water in individual housing chambers and kept in an incubator. Each housing chamber was inspected every 12h; during each inspection time, dead individuals were removed and diets replenished for survivors. Wing length of each carcass was measured and used to assess whether body size significantly influenced survivorship. Survivorship was assessed as the time from when an individual was put into a housing chamber to the time of death of the individual. Effects of diet and wing length on survivorship were assessed via ANCOVA.

RESULTS

Diet selection

Dye colour did not significantly influence the source from which mosquitoes fed (Fig. 4.2; χ^2 goodness-of-fit test (1, N = 49) = 0.18, p = .67). The selection pattern of mosquitoes when presented with a choice between an amino acid-free diet and a diet enriched with the amino acids, asparagine and glutamine did not display a significant selection of any diet type (Fig. 4.3; χ^2 goodness-of-fit (1, 37) = 2.2, p = .14 for the nectar (N) versus enriched nectar (EN) comparison, and χ^2 goodness-of-fit (1, 21) = 2.3, p = .13 for the honeydew (H) versus enriched honeydew (EH) comparison.

The melezitose content of diets seemed to influence the diet selection pattern of mosquitoes. Although the diets, which contained melezitose, also had more calories, the mosquitoes appeared not to forage from the higher calorie diet but rather the lower calorie ones (Fig. 4.4). Of six different paired choice experiments that investigated the effects of melezitose on diet selection, only two showed significant difference in the selection of one diet over the other, both cases being the selection of the lower calorie, melezitose-free nectar diet over the higher calorie, melezitose-containing diet. Significantly more mosquitoes fed on the concentrated nectar (CN) than honeydew (H) (Fig. 4.4; χ^2 goodness-of-fit test (1, N = 30) = 8.5, p = .003). Mosquitoes showed a significant selection for concentrated nectar (CN) over concentrated honeydew (CH) (Fig. 4.4; χ^2 goodness-of-fit test (1, N = 33) = 10.9, p = .001).

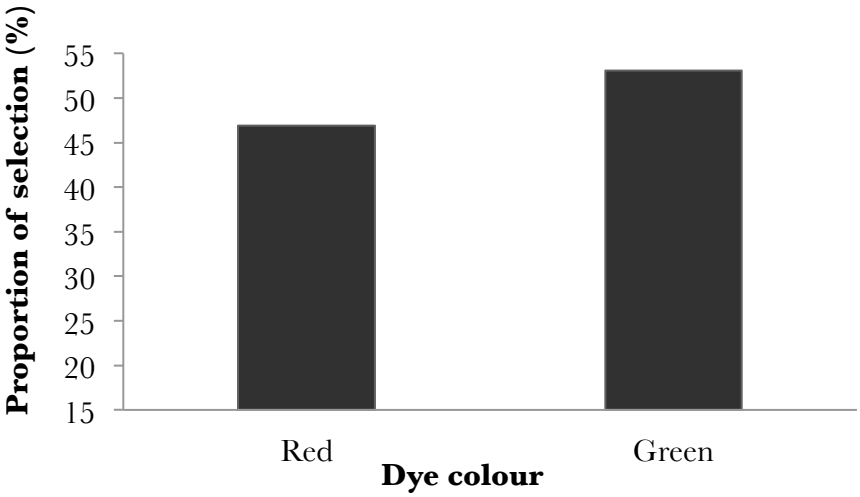


Fig. 4.2. Proportion of female *An. stephensi* mosquitoes that fed on 10% sucrose solution dyed with red or green colours. Dye colour did not significantly influence the selection. N = 49. χ^2 goodness-of-fit test $(1, N = 49) = 0.18, p = .67$

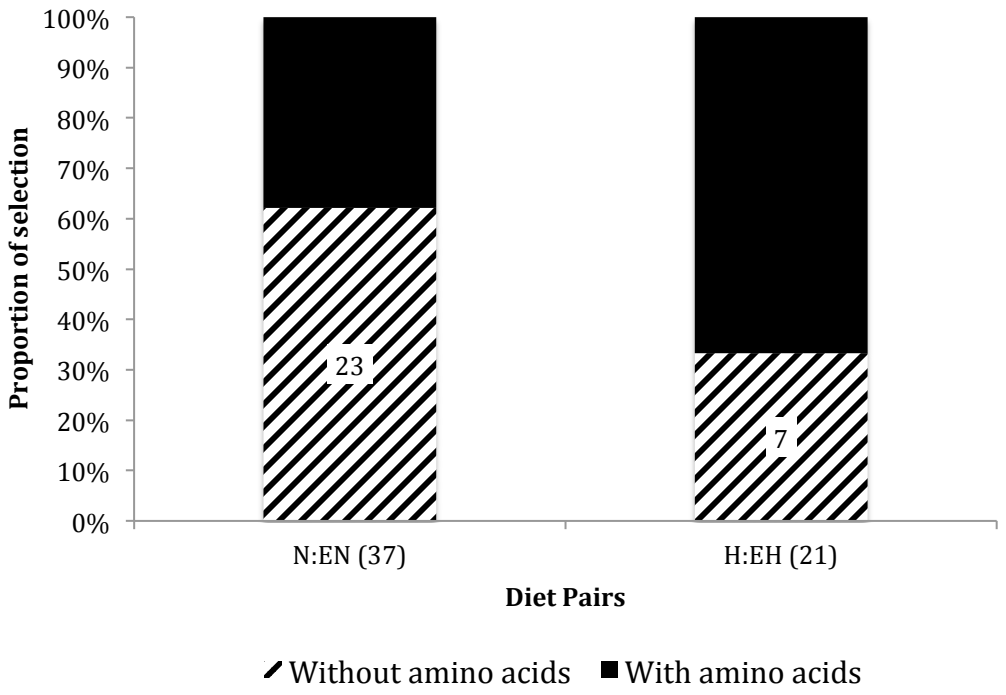


Fig. 4.3. Influence of amino acids on the diet selection pattern of female *An. stephensi* mosquitoes. N = nectar, EN = enriched nectar = nectar + glutamine + asparagine, H = honeydew, EH = enriched honeydew = honeydew +glutamine + asparagine. Glutamine and asparagine do not affect diet selection of the mosquitoes. Numbers in bracket = N. Numbers in columns = number of individuals that fed on the first diet in each diet pair. χ^2 goodness-of-fit (1, 37) = 2.2, p = .14 for the N versus EN comparison, and χ^2 goodness-of-fit (1, 21) = 2.3, p = .13 for H versus EH comparison.

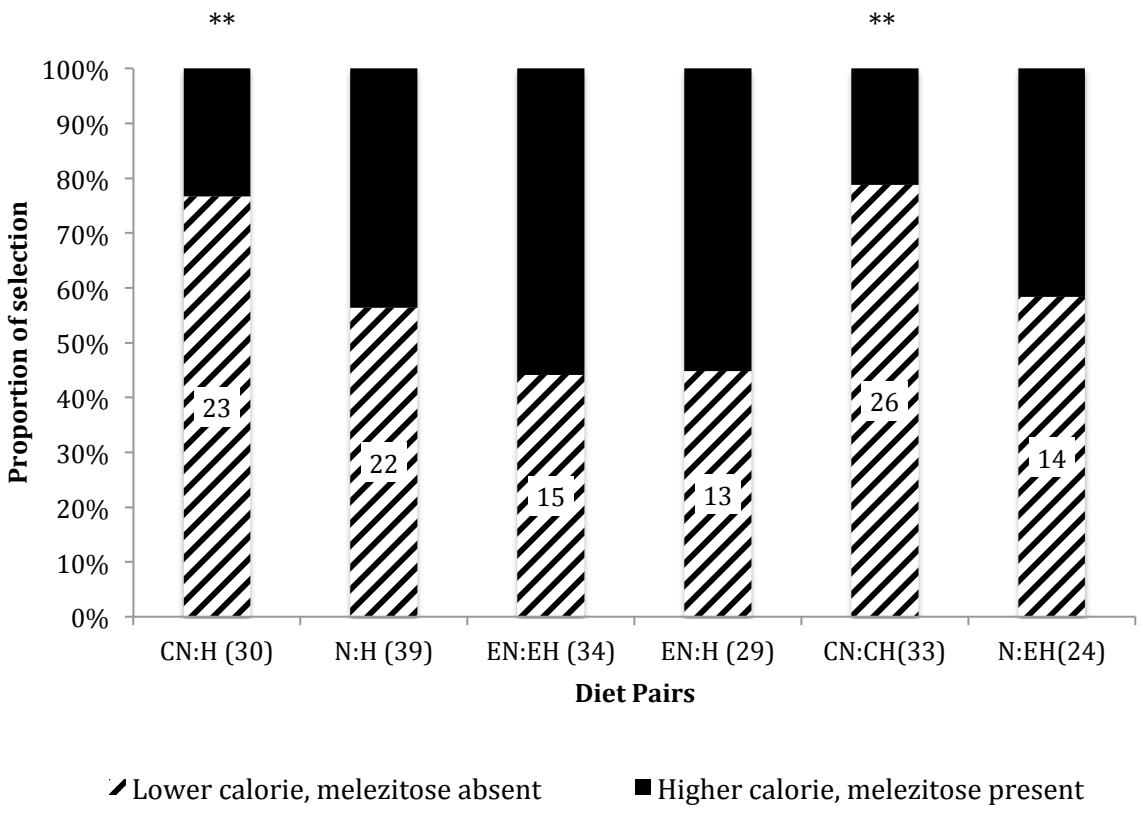
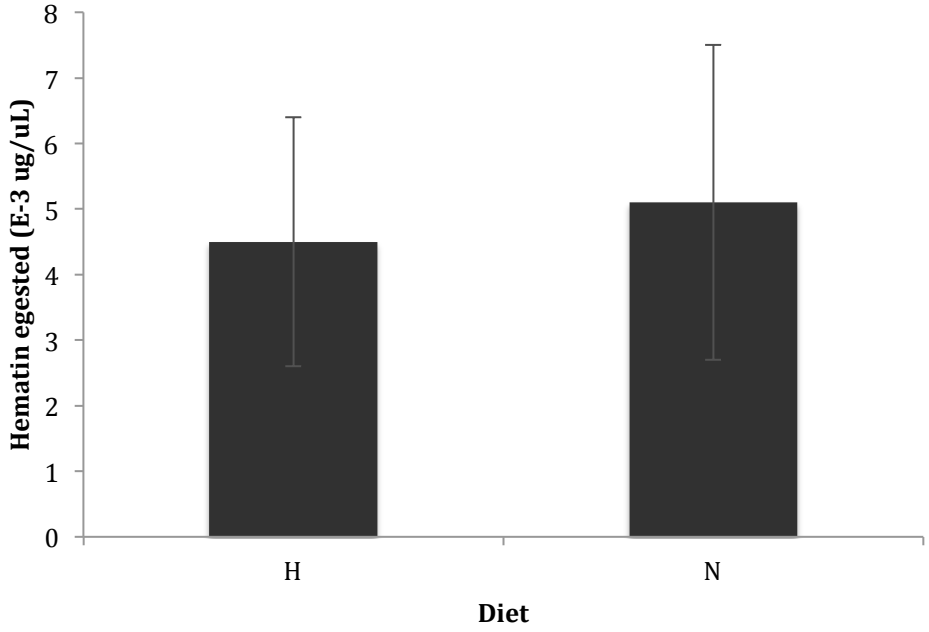


Fig. 4.4. Combined effects of calorie and melezitose content of sugary diets on the diet selection pattern of female *An. stephensi* mosquitoes. H=honeydew, N=nectar, EH=enriched honeydew, EN=enriched nectar, CH=concentrated honeydew, CN=concentrated nectar. Figures in brackets=N, ** $p < .01$, *** $p < .001$. Numbers in brackets = N, numbers in column = number of individuals that fed on first diet in each diet-pair.

Effect of sugary diet on amount of blood taken

An ANCOVA revealed no effect of the level of *Plasmodium berghei* infection in mice on the amount of blood taken by sugar-fed mosquitoes $F(1, 49) = .06$, $p = .82$ and, amino acid-fed mosquitoes $F(1, 41) = .77$, $p = .49$. There was no significant difference in the amount of blood ingested by mosquitoes that fed on nectar (N) and those that fed on honeydew (H) $F(1, 49) = .19$, $p = .66$. However, there was a significant effect of amino acid on the amount of blood taken by mosquitoes $F(2, 41) = 4.69$ $p = .02$. Post hoc analysis using Tukey's HSD indicated that glutamine-fed mosquitoes took significantly more blood than arginine-fed ($p = .03$) and asparagine-fed ($p = .02$) mosquitoes but no significant difference between the amount of blood taken by asparagine-fed mosquitoes and those that fed on arginine ($p = .10$).

(a)



(b)

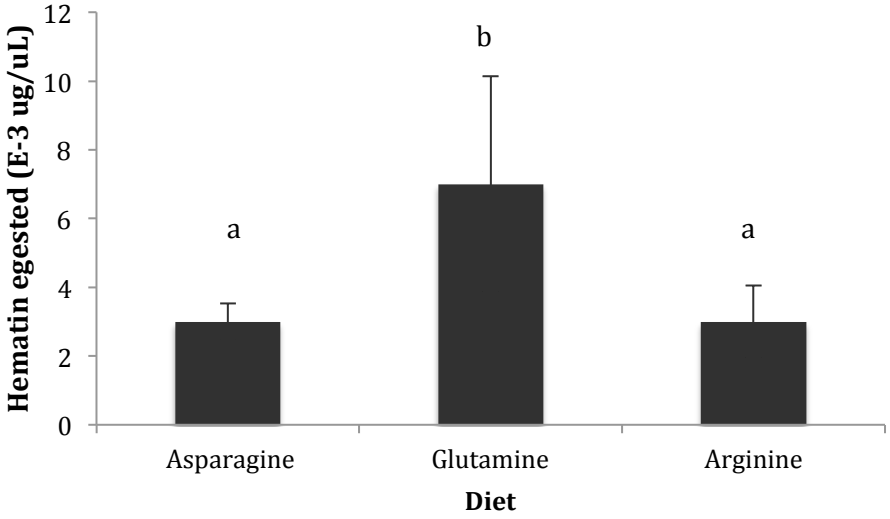
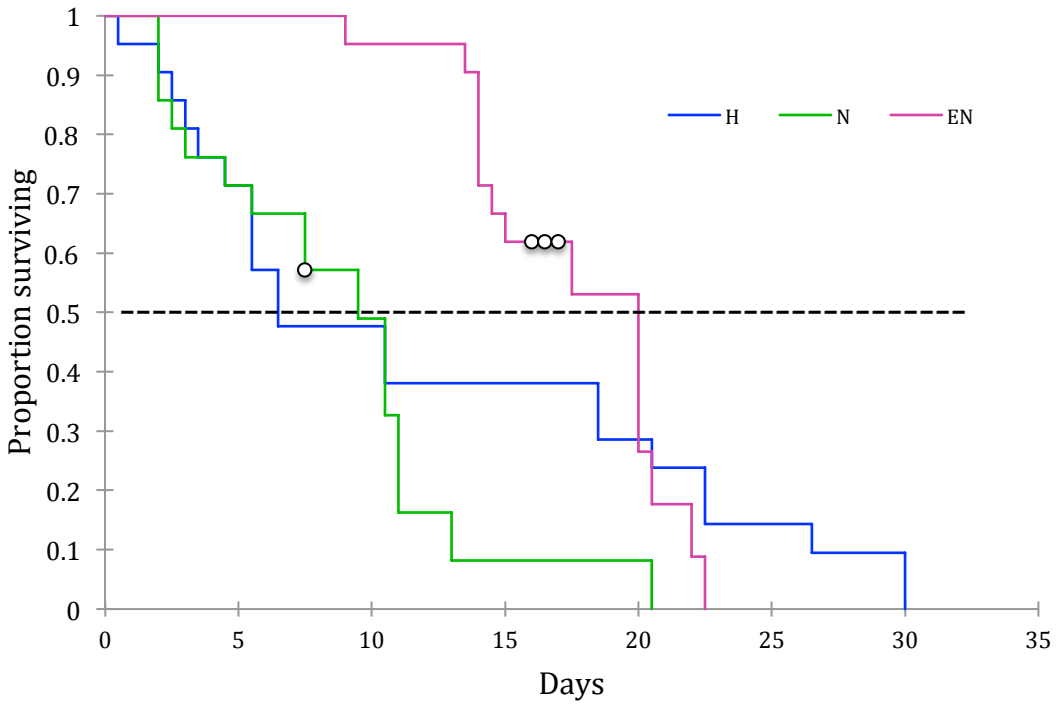


Fig. 4.5. Mean amount of blood ingested (estimated by amount of hematin egested) by *An. stephensi* mosquitoes after being allowed to feed on treatment diets for four days prior. (a) Sugary diets do not affect the amount of blood ingested, N = 25 in both cases (b) amino acid diets influenced amount of blood ingested. Bars with different letters are significantly different. N = 14 for each treatment, Error bars = 95% confidence interval.

Effect of sugary diet on survivorship

A Kaplan-Meier analysis of survivorship showed an effect of diet on the survivorship of female *An. stephensi* {(Fig. 4.6; Log-rank test (2, 10.1), $p = .007$)}. Follow up pair wise comparisons showed that mosquitoes that were fed with enriched nectar (EN) ($M = 16.7$, $CI = 1.99$) lived longer than those fed with nectar (N) (($M = 7.6$, $CI = 2.67$) or honeydew (H) ($M = 6.9$, $CI = 2.77$) $p < .0001$ for both comparisons but no significant difference in survivorship for mosquitoes fed on nectar versus those fed on honeydew (p = .67). There was no significant effect of wing length on survivorship {F (1, 44) = 1.71 $p = .20$}.

(a)



(b)

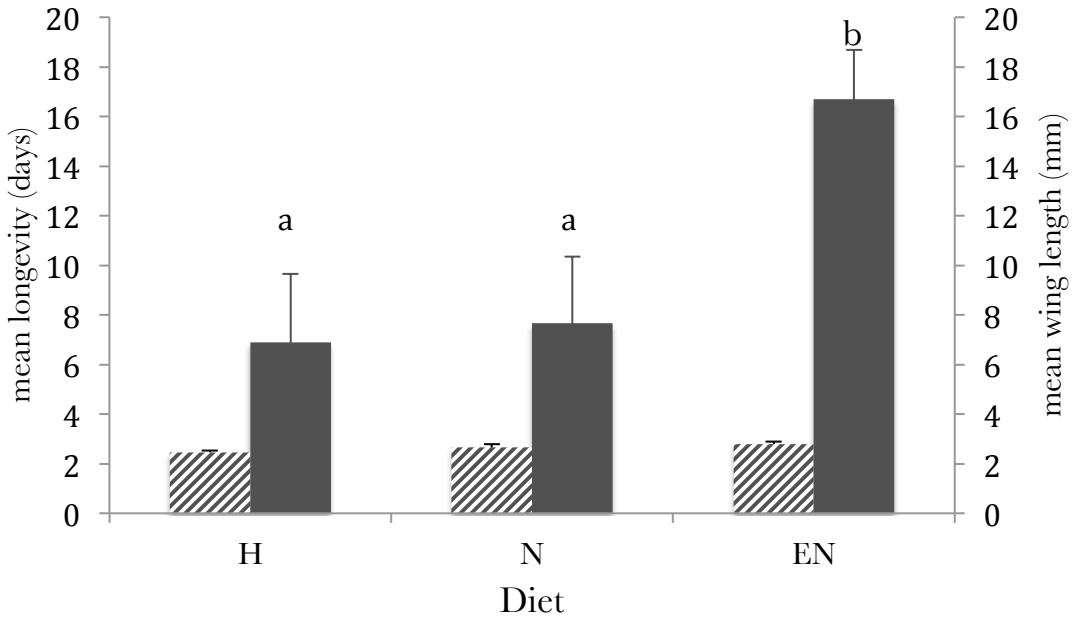


Fig. 4.6. Survivorship of *An. stephensi* mosquitoes fed on sugar diet preparations. (a) effect of diet on the survival distribution of mosquitoes, in each case, open circles represent censored observations. (b) mean longevity and mean wing length of the mosquitoes. Hatched columns = mean wing length, solid column = mean survivorship. Error bars = 95% CI, treatment pairs with different letters have significantly different effects on longevity.

DISCUSSION

There was no effect of red or green colour on *An. stephensi* selection pattern (Fig. 4.2). This outcome is exactly as reported by Lindh et al. (2006) in feeding experiments with *An. gambiea*. These outcomes suggest that colouring sugar solutions with these colour combinations at least, could be a standard procedure in diet selection/preference studies involving dipterans. Besides the simple and reliable (due to fact that mixed feedings can easily be seen) nature of this procedure, its principal advantage is its cost effectiveness compared to alternative methods such as stable isotope labeling and subsequent mass-spectroscopy analysis (Lindh et al., 2006).

Diet selection work by Lindh et al. (2006) involving *Ae. Aegypti, An. arabiensis* and *An. gambiae* indicated a significant "preference" for diets with the higher concentration of sugar. However, in the present study, the foraging behaviour of the *An. stephensi* study did not follow the outcomes obtained by Lindh et al. (2006). Generally, the mosquitoes fed from lower calorie diets than the ones with higher calories (which were also the more concentrated in the pairs) (Fig. 4.4). These outcomes did not conform to the predictions of the static category of optimal foraging theory models (Brown, 1993; MacArthur & Pianka, 1966). Briefly, this model predicts that in situations where there is no variation in distribution in time and space of alternative food sources, organisms will forage from food the most profitable food source (defined as the total calorie content per milliliter of a diet). Calorie content in diets used in the present study represent physical calories, which were calculated from the heats of combustion of the individual nutrients (see Chapter 2). Thus, the caloric value of a diet does not necessarily equal the amount of energy that an organism that feeds on it can extract from it.

Two factors may actually be more important in assessing whether mosquitoes forage from the most profitable food source, and may account for the 'non-optimal' nature of the foraging behaviour of *An. stephensi*. First, the volume of sugary solutions imbibed by an individual will determine how much calories can be extracted from the food source. Therefore an organism may need to feed more frequently from a low calorie food source, and take in higher volumes of the diet per feeding time, in order to maximize its caloric intake with the concomitant need to osmoregulate. Conversely, the same individual may take smaller volumes, and, feed less frequent on a high calorie diet. Another factor that may influence the profitability of a diet is the bioavailability of the nutritional components of that diet. Bioavailability in this sense may be defined as the proportion of dietary nutrient that is digested, absorbed and metabolized by mosquitoes (Srinivasan, 2001).

In the present study, *An. stephensi* selectively fed on the higher calorie diet in only two out of six trials. However, all the high calorie diets were also more concentrated (than the alternative), and contained the trisaccharide melezitose. It is not known whether mosquitoes show different gustatory response to the individual sugar and amino acid components of the treatment diets but it reasonable to suggest that *An. stephensi* mosquitoes are capable of detecting the presence of melezitose in the higher calorie diets. Perhaps the mosquitoes avoided feeding on the high calorie, high concentration diets because they are unable to digest the melezitose contained in those diets, implying that melezitose may not be biologically available to female *An. stephensi*. It has been established that mosquitoes are capable of discriminating between sugary food sources; generally preferring more concentrated sugars to dilute ones (Lindh et al., 2006; Manda et al., 2007). Manda et al. (2007) reported that *An. stephensi* "preferred" diets with high

glucose and fructose content. The two instances of significant selection for a diet in the present study involved the concentrated nectar diet, which was the diet with the most concentration of glucose and sucrose among all the treatment diets (see Table 2.1). Perhaps the glucose and fructose concentration of diets is more important in determining the foraging behaviour of *An. stephensi* mosquitoes than total solute concentration or total calorie content of diets.

The absence of mixed feeds in a total of 247 mosquitoes tested, suggests that the mosquitoes did not explore the other food source in a pair when they started to feed on one. Lindh et al. (2006), however, found 'a few' incidents of mixed feeding. Friend, (1978) suggested that once an insect is engaged in a particular feeding mode, the sensory perception of the individual will be biased towards stimuli specific to that mode. This may account for the rare nature of mixed feeding in diet selection experiments in the work of Lindh et al. (2006) and this present report. A major design weakness in the present study was the inability to assess accurately the volume of sugary solution ingested by each individual. Although I scored the level of engorgement (i.e. volume of diet ingested) by individuals on a five-point hierarchical scale, the high variability in this measure even within a single treatment group made this approach unreliable, as it tended to be too subjective. Subsequent work on this subject will benefit from a clear delineation of engorgement levels and a plan to assess how this influences the foraging behavior of mosquitoes.

Gary & Foster (2004) reported that *An. gambiae* take sugar meals from plant and honeydew sources in the immediate vicinity of human dwellings before seeking blood. According to Ma (2010) a crop fully filled with a sugar meal will make taking a replete blood meal afterwards impossible since both the crop (where sugar is stored) and the

midgut (where blood is stored) share the same limited abdominal cavity. Therefore, during sugar feeding before blood feeding, mosquitoes should take in less amount of sugar to leave room for the distended midgut when they eventually blood feed. In the present study, the amount of blood taken by mosquitoes was not influenced by the sugary food they had previously fed on (Fig. 4.5a). However, amino acid preparations, specifically the glutamine diet, influenced amount of blood taken (Fig. 4.5b).

Since ingested glutamine is converted to glutamate in dipteran tissue, which is further metabolized into γ-amino butyric acid (GABA) (Dowton & Kennedy, 1986), and GABA is known to have antifeedant properties in phytophagous insects (Bown et al., 2006; Gonzalez-Coloma et al., 2002; Shelp et al., 2006), it is possible that *An. stephensi* that fed on glutamine in the present study actually fed on relatively small amounts due to the antifeedant effect of GABA synthesized from ingested glutamine, and therefore, were able to feed on more blood given that they had more capacity to store ingested blood. Richardson et al. (2010) reported that the presence of the glutamate decarboxylase (the enzyme that metabolizes glutamate to GABA), in *An. gambiae* and all insects whose genome are currently available, which includes *An. stephensi*. It is also likely that the glutamine diet did not elicit a feeding response from the mosquitoes, thereby making them more likely to feed on more blood. This explanation is however weakened by the fact that the present study did not investigate the differences in the ability of nutrient components to elicit feeding responses from the mosquitoes, and the probability of no difference in the ability of glutamine and asparagine to elicit a feeding response from the mosquitoes. The black fly work in the present study (Chapter 3) and work by Goldrich (1973) seem to support this assertion.

The present study provides further evidence that sugar feeding is essential to the survival of mosquitoes. Female *An. stephensi* mosquitoes fed with distilled water had the lowest survival rate. This observation is similar to that reported by Manda et al. (2007). In that study, the investigators found that female *An. gambiae* lived longest when they fed on those diet that had high glucose and fructose content, and suggested that incidents of mosquitoes feeding on diets with low glucose and fructose, may have been due to the presence of other nutrients present in those diet sources. The other nutrients referred to by Manda et al. (2007), may have been amino acids. In the present study, these sugars alone did not enhance the survivorship of the mosquitoes but needed the addition of glutamine and asparagine to significantly influence survivorship. Glutamine and asparagine are therefore important to mosquito survival.

CHAPTER 5:

GENERAL DISCUSSION

The present study demonstrates that dye colour does not influence the diet selection pattern of female *S. venustum/verecundum* blackflies and female *An. stephensi* mosquitoes. When presented with two 10% sucrose solutions dyed either red or green at the same time, black flies and mosquitoes did not display a significant selection of any colour. In both insect groups, the colours red and green was not important in predicting diet choice. These colours could be used in further diet selection studies, involving these insect groups. This is further support for the suggestion by Lindh et al., (2006) that colouring food dyes is an efficient, reliable, and cost effective alternative to isotope labeling of diets and subsequent mass-spectroscopy analysis to assess diet selection. In addition, the simplicity and relative ease of this procedure makes it suitable for field or semi-field experiments; it does not require any special equipment, or technical skills apart from the assessment of which colour combinations to use for the particular insect group.

The present study presents evidence of the ability of mosquitoes and black flies to discriminately feed from certain food sources. It is the first attempt at establishing the diet factors that influence nonrandom foraging behaviour in these medically important dipteran families. Black flies demonstrated a diet selection pattern that suggests that they forage more frequently on the more profitable diet in a diet pair, i.e. they fed from those diets that either provided more calories per unit ingested, or the one that provided a greater variety of nutrients, where the diet options available had similar calorie content. Mosquitoes, on the other hand, did appear to forage indiscriminately from the diet sources. Further, the present study, demonstrates for the first time, that *Simulium*

venustum/verecundum species do not discriminate in their ability to feed on the common sugar and amino acid components of nectar and honeydew, but differentiate between sugary food sources principally by the total calorie content of those diets. Whereas other research (Gary & Foster, 2004; Manda et al., 2007), indicate that Anopheline mosquitoes are capable of selective sugar feeding, this is the first study that investigates the effects of sugar and amino acid components of sugary food sources on the foraging decisions of the medically important *Anopheles stephensi*.

The foregoing discussions on the foraging decisions of the dipterans used in the current study is based on the primary assumption that the insects forage in such a way as to maximize their caloric intake, and consequentially, increase their relative fitness (Mitchell, 1982, MacArthur & Pianka, 1966). Whereas this assumption is sufficient in the ecological and evolutionary sense, it falls short when the digestive physiology of the consumers and the potential interactions of the nutrient solutions are considered. The consumers' digestive physiology determines how much of the physical calories is liberated and made available for use upon digestion. Thus the relative digestibility of individual nutrients and the various combinations (as in the diets) need to be studied to ascertain whether diet selection behaviour of black flies and mosquitoes is influenced by their respective digestive physiologies.

Previous work has established that black flies and mosquitoes utilize different sugary food sources, and demonstrate to some extent, selective feeding (Burgin & Hunter, 1997a; Gary & Foster, 2007; Manda et al. 2007). However, those researchers used natural plant and homopteran sources of sugary food in their feeding experiments, and this may complicate the interpretation of outcomes. The benefits of using these naturally obtained food sources in feeding experiments are obvious; the most import

being that they are the diets that are available to in the study organisms in their natural habitat and, thus, outcomes of feeding experiments can be confidently extrapolated to explain and predict feeding behavior in another ecosystem.

However, the use of naturally occurring sugary food sources in studies presents two main problems: first, the occurrence of non-nutrient inclusions such as alkaloids, glycosides, and phenolic substances in natural nectars for example, interact with the gustatory responses of foragers on these sugary foods (Baker, 1977), second, naturally occurring nectars and honeydews show inter- and intraspecific differences in sugar and amino acid profiles (Baker & Baker, 1977; 1986; Yao & Akimoto, 2002; Winkler, et al. 2005; Woodring et al. 2004) therefore making the replication of experiments involving natural nectars and honeydews extremely difficult to replicate. For these reasons, artificially prepared diets, made from the main nutritional components of nectars and honeydews present a better opportunity to obtain baseline information on how diet-specific factors such as concentration and nutrient composition influence diet selection. Further, the use of artificial diets as opposed to natural ones make the interpretation of observed ecological benefits of sugary food feeding simple and reliable as observed differences can reliably be accounted for by the differences in the treatments given that other confounding factors are controlled for.

In the present study, sugar feeding was found to influence the survivorship of both families of flies. In both black fly and mosquito groups, individuals who fed on water only, lived the shortest among all treatment groups. Although no sugar diet was able to significantly extend the survivorship of black flies more than any other sugar diet, amino acids appeared to be important to the survival of mosquitoes because individuals who were provided with nectar diet enriched with the amino acids, glutamine and asparagine

lived longest among three treatment groups. The outcome in the mosquito test needs to be interpreted cautiously because the effect of amino acids was not investigated in the honeydew-fed flies due to resource constraints.

The effect of prior sugar feeding on the amount of blood ingested was investigated in the mosquitoes only. In that experiment, glutamine was the only dietary nutrient that was determined to affect the amount of blood taken by *An. stephensi*. Individuals that fed on glutamine preparations took significantly more blood than all other groups fed the other diets. This poses the question of whether glutamine might play a critical role in egg maturation, such that increased glutamine consumption increases blood feeding to accommodate a higher egg "load." It also suggests that consumption of honeydew and, to a lesser extent, nectar, would increase blood feeding by mosquitoes and, thus, increase the risk of parasite infection.

In black flies (*S. rugglesi*), the number of eggs in the ovaries (i.e. developed) was not influenced by the diet on which they fed. Diet however influenced the number of *L. simondi* oocysts found on the midgut wall, with fewer oocysts observed on honeydew fed flies.

Taken together these observations (in the black flies and mosquitoes) suggest that sugary diet feeding influences the competence of these dipteran vectors to transmit the parasites they are associated with, with important implications in the control of parasites transmitted by these flies. It has been established that sugary food feeding influences the energetic budget and survival of the African malaria vector *An. gambiae* (Gary & Foster, 2004). If a sugary food source increases the survival of individual vectors in a population, the number of blood feeding cycles (and potentially the amount of blood taken) by reproductive individuals within that population is likely to increase, which may in turn,

increase the probability that a pathogen may be picked up from a host or transmitted to the host (Anderson, 1987; Foster & Taken, 2004). The present study has presented evidence that suggest that sugary foods reduce the level of parasite infection in dipteran vectors. Thus, whereas sugary food feeding has potential anti-parasitic benefits to the vector, concomitant effects on other measures of vector fitness can negate parasite control benefits. Therefore, the use of sugary diets as ecological manipulation tools to control vector-borne diseases must be plan carefully with due consideration given to the counteracting effects of sugary food feeding on vector fitness.

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