NATURAL SUGARS DETECTED IN THE CROPS OF TWO SPECIES OF HORSE FLIES (DIPTERA: TABANIDAE) AND THE HYDROLYSIS OF SUGARS IN THE CROP AND IN SALIVARY GLAND HOMOGENATES

By

KENNETH LYNN HOPPE II

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Oral Roberts University

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Thesis Approved:

Russell Wright
Thesis Advisor

William A. Drew

Jack W. Dillwith

Norman N. Durham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

The large and cosmopolitan family Tabanidae (Diptera) includes avidly bloodsucking flies known by a variety of names, the most common being horse flies, deer flies, clegs, and mango flies. Tabanids are among the most important pests of man and livestock in the world. Their role as biological and mechanical vectors of disease agents of man and animals was reviewed by Krinsky (1976). In North America, their greatest impact is on cattle through direct attack causing blood loss and energy loss from irritation (Hollander and Wright 1980a). Anautogenous tabanids are obligatory pest because of their need for a blood meal for the completion of the first and subsequent ovarian cycles (Leprince and Lewis 1986). Perich et al. (1986) reported heifers were 16.9% less efficient in feed utilization and gained less weight under tabanid attack than protected cattle. Wright et al. (1986) reported 64 tabanid species in Oklahoma. In northcentral Oklahoma, Wright et al. (1984) reported seven genera and 23 species of tabanids and that six of these species constituted 97.7% of the total specimens captured from 1978 to 1981. The most abundant species was Tabanus abactor Philip, with an estimated

average number of host-seeking females per hectare of 2,225 and 1,651 for 1982 and 1983, respectively (Cooksey and Wright 1989). The general energy requirements of tabanids for survival and flight are supplied by carbohydrates obtained from floral nectars or other sugar sources (Hocking 1953, Leprince et al. 1983). The relationship of host-seeking and nectar-feeding have been studied in the mosquito as it relates to reproduction and disease transmission (Klowden 1986, Foster and Eischen 1987). Because of the importance of sugar in the survival of tabanids, the purpose of this study was to gather further information on the sugar feeding behavior of T. abactor and Hybomitra lasiophthalma (Macquart) in northcentral Oklahoma.

The initial approach of the study was to determine the sugars contained in the crops of field collected flies to possibly indicate what family of plants the flies were feeding on in nature and to identify potential plant sugar sources. However, after examining the sugars in the crops, concerns arose about the possibility of digestive enzymes changing the sugar composition in the crop. The consequent objective was to determine if the sugars in the crop were being digested and if so, the source of the digestive enzyme.

The final objectives of the study were to 1) determine the natural crop sugars, 2) determine if digestion of sugars was occurring in the crop, 3) adapt a high performance thin

layer chromatography technique for small amounts of sugar to use in objectives one and two.

CHAPTER II

LITERATURE REVIEW

The Need of Sugars for Survival in Tabanids

The association of both male and female tabanids with plants is well documented (Olsufjev 1937, Hocking 1953, Downes 1958, Roberts 1967, Lall 1970, Pratt and Pratt 1972, Magnarelli and Anderson 1977, Magnarelli et at. 1979, Magnarelli 1985a). Various species of horse flies can survive for a month in the laboratory when fed sugar solutions but live for only a few days when maintained on water (Wilson 1967, Wilson and Lieux 1972, Leprince et al. 1983, Magnarelli and Burger 1984). Van Handel (1965, 1984) reported that female mosquitoes accumulate a large amount of fat from sugar meals and use these reserves for survival at rest and use mainly glycogen for flight. Male mosquitoes synthesize only a very minute quantity of fat and small reserves of glycogen. In contrast, Auroi and Briegel (1985) reported that horse flies do not synthesize net reserve material in substantial amounts, either as fat or glycogen, and concluded that frequent feeding, several times a day, on carbohydrate sources is necessary for survival. Magnarelli (1985a) reported that blood-seeking Crysops fuliginosus Wiedemann had low energy reserves, indicating that the

simple sugars obtained from various food sources were used to provide immediate energy for flight and survival rather than converted into reserves, such as glycogen and triglycerides.

In tabanids, the crop is the main destination of sugar meals, while blood meals and water are directed to the midgut (Hocking 1953, Bosler and Hansens 1974, Stoffolano 1983). Stoffolano (1983) proposed that female T. nigrovittatus Macquart has the same three modes of feeding as reported by Friend (1978, 1981) for female mosquitoes. The three modes are (1) a drinking mode where small quantities of water are directed to the midgut, (2) a nectar or sugar-feeding mode where fluids are dispatched to the crop, and (3) a blood-feeding mode where the meal is directed to the midgut. Magnarelli and Anderson (1981) suggested that there may be two strategies for sugar acquisition and energy production: (1) a large intake of nectar and a slow release of sugar from the crop to provide energy or (2) intermittent ingestion of small sugar meals throughout the flight period and immediate metabolism.

Magnarelli (1979) reported that blood-seeking and resting female Aedes cantator (Coquillett) and A. sollicitans (Walker) contained fructose throughout daylight hours, but that the average percentages of fructose-positive specimens were significantly greater between 2000 and 2100 hours. He reported that the percentage of fructose-positive male specimens exceeded 55% in each species and were

consistently higher than those of females between 0800 and 1700 hours. Reisen et al. (1986) reported that Culex tarsalis Coquillett females ingested sugars in the early morning (0200-0600 hrs) after depleting their energy reserves during the first half of the night, apparently ingesting fructose after blood feeding. Magnarelli et al. (1979) reported that T. nigrovittatus and C. fuliginosus blood-seeking females were fructose-positive during the entire day (0900-2000 hrs), indicating that female tabanids may require nectar throughout the day. Their observations of male and female C. fuliginosus on swamp rose and yarrow in Connecticut between 0700 and 2000 hours showed a biphasic periodicity. In Europe, Kniepert (1980) reported there was a decrease in sugar-feeding by the females of several species of tabanids associated with an increase in blood-feeding through the summer.

Downes and Dahlem (1987) proposed that Diptera originally depended on honeydew as an energy source and that this explains their "dancing behavior"; their attraction to small, shiny objects; and the presence of sugar receptors on the tarsi. Honeydew feeding of salt marsh horse flies has recently been reported by Schutz and Gaugler (1989).

Plant Sources of Energy and Nutrients

Floral nectar consists mainly of sucrose, glucose, and fructose (Wykes 1952, Percival 1961, Baker and Baker 1982). Baker and Baker (1982) reported other sugars that occur in

minute amounts in nectar as arabinose, gentiobiose, galactose, lactose, mannose, maltose, melibiose, trehalose, melezitose, and stachyose. Some of the minor sugars are present in fresh nectar but their concentrations may increase in nectar that stands as liquid due to the breakdown of other sugars (Mauritzio 1959). In addition to sugar and water, numerous organic chemicals such as amino acids, proteins, lipids, and organic acids have been reported from nectars (Baker and Baker 1982).

Floral nectar is one of the rewards that angiosperms make available in their flowers for animals that may be useful as pollen-vectors, such as insects, birds, and bats (Baker and Baker 1982). The chemical constituents of floral nectars, sugars and amino acids, vary according to the flowers (Baker et al. 1978). Flowers visited by flies tend to be hexose-rich, verses sucrose-rich (Baker and Baker 1983b) and concentrations of the sugars are higher than those taken in by long-tongued insects such as Lepidoptera (Baker and Baker 1983a).

Clues other than natural sugars are important in the selection of host plants by insects. Magnarelli (1979) indicated peak bloom and high density of the nectar source plant were important in determining which nectars were fed upon by tabanids. Vargo and Foster (1982) reported that volatile chemicals from flowers attracted mosquitoes.

Extrafloral nectary glands may be located on leaf laminae, petioles, and the rachids, bracts, stipules,

pedicels, fruit, etc, varying with plant taxa (Rogers 1985). Extrafloral nectars are about 97% sugar and most have a full complement of amino acids, plus a number of fatty acids (Rogers 1985). The constancy of extrafloral nectar during adverse environmental conditions may be the greatest asset of extrafloral nectar as a source of nutrients for insects when other food sources are either in short supply or lacking (Rogers 1985).

Another source of carbohydrate is the liquid exudate that seeped from the tunnels of the carpenter worm in oak trees in Louisiana. Roberts (1967) reported the liquid exudate was fed upon by T. atratus F., T. americanus Forster, T. proximus Walker, and T. stygius Say during a dry period.

In the attempt to determine the carbohydrate sources for tabanids in the field, it was assumed that the presence of pollen grains in their digestive tract would furnish presumptive evidence that they had fed on nectar in the flowers of the species involved or on honeydew or various exudates of the plants, the pollen having fallen or blown into the material in question (Wilson and Lieux 1972). Also, the acquisition of pollen with nectar might be significant because proteins and amino acids are released from certain pollen when incubated in a sucrose solution (Stanley and Linskens 1965, Linskens and Schrauwen 1969).

Hocking (1953) listed eight flowers visited by tabanids and indicated that five were "based on pollen grains

collected from the insects" and the others were from direct observation. Wilson and Lieux (1972) examined the midgut and crop of 11 Tabanus species and H. lasiophthalma in Louisiana. From tabanids collected in alluvial areas, 27 pollen types were identified, and 21 pollen types were identified from tabanids collected in salt marsh areas. The most frequently observed pollen in tabanids collected in alluvial areas was that of oak (Quercus spp.), and a relative abundance of pollens of pines (Pinus spp.), and the composite family (Compositae). From the number of pollen types found in the guts of the tabanids they examined, Wilson and Lieux (1972) concluded that tabanids seek carbohydrate meals from a variety of plant sources. They stated that oak pollen in T. sulcifrons Macquart that emerge late in the summer indicates that some tabanids are probably feeding on tree sap or some other exudate in which pollen had been trapped months earlier when it was being shed. They also speculated that the large quantities of grass pollen found in T. lineola hinellus Philip in the salt marsh may indicate that this species utilizes pollen as one source of carbohydrates and protein, since grasses have virtually no nectar.

Bosler and Hansens (1974) did not detect pollen grains in the guts of 379 T. nigrovittatus, but noted pollen was present on the cuticle and body hairs. Magnarelli et al. (1979) found pollen grains in 42 of 150 T. nigrovittatus, C. fuliginosus, and C. atlanticus Pechuman. Nearly 80% of 119

pollen grains recovered were in the two Crysops species. Thirteen out of 25 T. nigrovittatus contained 51 pollen grains. They concluded that tabanids probably ingest pollen in nectar, but with the low number of pollen grains present internally, it was difficult to conclude that they were directly acquired from specific host plants.

In Quebec, Canada, Leprince et al. (1983) examined 22 male H. sodalis (Williston) externally for pollen grains and found 40% had pollinia. They reported the most common and abundant were eight nectariferous plants with large inflorescence, which are probably used as landing platforms.

"Honeydew" is the liquid droplet excretion from the alimentary tract, as released through the anus of aphids, coccids, and many other plant sucking insects (Auclair 1963). Studies have demonstrated that fresh honeydews are usually complex mixtures of a large variety of chemical compounds, including several sugars, amino acids and amides, organic acids, alcohols, auxins, salts, etc. as reviewed by Auclair (1963). Fructose, glucose, and sucrose are general constituents of honeydew, together with a few oligosaccharides such as melezitose and others of the glucosucrose (fructomaltose) series. Walters and Mullin (1988), report that osmotic pressure due to high sucrose concentration in the diet of potato aphids results in significantly higher levels of melezitose and six additional oligosaccharides being recovered from the honeydew. Aphid honeydew fed on by C. fuliginosus consisted of glucose,

sucrose, and small amounts of fructose (Magnarelli et.al. 1979). Kniepert (1980) cites three other reports of tabanids feeding on aphid honeydew, one of dry honeydew from a leaf.

Sugar Detection in Tabanids

Methods used to determine nectar sugars in tabanids have been the cold anthrone test and thin layer chromatography (TLC). The cold anthrone test (Van Handle 1967) has been used for quantitative and qualitative determination of fructose and oligosaccharides that yield fructose upon acid hydrolysis. The cold anthrone reagent hydrolyzes the sugar and the anthrone reacts with the fructose moiety producing a green-blue color and the optical density of the solution can be used for quantitative results. The cold anthrone test is quick and simple, especially for qualitative results which can be obtained in the field. The insect is crushed with a glass rod in a test tube or a ceramic spot plate containing the reagent mixture and observed for thirty minutes to one hour for color development. The cold anthrone test has been used to detect fructose in mosquitoes (Van Handel 1972, Bidlingmayer and Hem 1973, Magnarelli 1978, 1979, 1980, Reisen et al. 1986, Andersson and Jaenson 1987), biting midges (Magnarelli 1981), and tabanids (Magnarelli and Anderson 1977, 1981, Kniepert 1980, Leprince et al. 1983, Magnarelli 1985b, Leprince and Lewis 1986). However, the percentage of the

tested population that has fed on sugars containing fructose is always higher than the anthrone test indicates compared with TLC (Bidlingmayer and Hem 1973, Magnarelli and Anderson 1977, Van Handel 1984).

According to Van Handle (1967) the cold anthrone reagent reacts with fructose, inulin, sucrose, melezitose, and raffinose. These sugars and other sugars with the fructose moiety give a positive reaction using the cold anthrone, but this does not give any indication of what sugars the fructose moiety originated from. Fructose, glucose, and sucrose are the major sugars in nectars and fruit juices but melezitose and other oligosaccharides are present in some aphid honeydews. The cold anthrone test fails to indicate specific sugars the insect has fed on.

The only TLC technique used for analyzing the sugars of tabanids was the one described by Magnarelli and Anderson (1977). For this method, the fly is crushed by a glass rod in 95% ethanol on a ceramic spot plate and the ethanol is collected and spotted on a TLC plate. The results obtained were used to indicate sugar-positive. Magnarelli and Anderson (1981) reported the detection of trace amounts of sugars with Rf values similar to maltose and melezitose. Magnarelli and Anderson (1977) used a standard sugar concentration of 1% and spotted 1 μl.

If the crop is used only for storage, then the sugars fed on would be the sugars in the crop. But, if there is an enzyme introduced from the saliva or midgut, then the sugars

in the crop would be hydrolyzed and an accurate picture of what the specimen had fed on would not be obtainable.

Alimentary Canal and Salivary Glands

The alimentary canal of the horse fly, Tabanus, is typical of the higher Diptera in the elaboration of the crop as a diverticulum of the foregut. The crop duct arises from the foregut immediately anterior to the cardia. It runs posteriorly from the thorax to the abdomen where it ends in a blind bilobed sac, the crop. The wall of the crop is composed of a simple layer of very small flattened cells, external to which there are many small muscle fibers arranged in an irregular network (Patton and Cragg 1913, Hocking 1953, Lall 1970).

The crop is the main destination of 1 M sucrose in T. nigrovittatus (Stoffolano 1983) as with the stable fly (Lee and Davies 1979, Venkatesh and Morrison 1980), mosquito (Clements 1963), and blow fly (Dethier 1969, 1976). The sugar solution in the crop is then transferred to the midgut in small amounts depending on the concentration of the sugar.

The salivary glands of many Diptera contain digestive enzymes. Hematophagous Diptera and blow flies have various carbohydrases in the salivary glands and in the digestive tract. Gooding (1975) reviewed the different digestive enzymes reported in hematophagous arthropods. Culex tarsalis is reported to have an invertase, maltase, and

melezitase in the salivary glands (Schaefer and Miura 1972). The blow fly, Calliphora erythrocephala Meigen, has an α-glucosidase, an α-galactosidase, a β-fructofuranosidase, a β-glucosidase, and an amylase in the saliva (Evans 1956, Hansen Bay 1978a). Hansen Bay (1978a, b) showed that saliva containing carbohydrases is secreted when C. erythrocephala feeds on sucrose, and this mixture of saliva and sugar goes into the crop, but such enzymes have not been reported in the salivary glands of horse flies.

A large amount of information is available on the morphology, physiology and feeding behavior of the blow flies, Phormia regina Meigen and C. erythrocephala. The general structure and behavior of the blow fly and the horse fly are similar. Chapman (1985) has reviewed the regulation of the passage of food through the gut of P. regina. When P. regina feeds on sugar the food goes directly to the crop and a full meal is passed to the midgut over the next 80 hours. The use of the crop as a reservoir enables the insect to utilize ingested food most effectively; absorbtion and digestion of substances more complex than monosaccharides, would be much less efficient if food was pumped directly to the midgut (Chapman 1985). Thomson and Holling (1975) described the mechanisms of crop emptying in detail, and Gelperin (1966) and Thomson (1975) describe the control of crop emptying.

Sugars and Carbohydrases

Carbohydrate is the form in which energy is most accessible to an animal (Chapman 1982). Most insects require an exogenous source of carbohydrate for flight, the most energy demanding of all life activities (McFarlane 1985). Oligosaccharides comprise a large and important class of polymeric carbohydrates which are found either free or in combined form. In structure, the oligosaccharides are composed of relatively few monosaccharide residues joined through glycosidic bonds which are cleaved by acid or enzyme hydrolysis to yield the constituent monosaccharides (Pazur 1970). The prefixes α- and β- are references to the hydroxyl group on the anomeric carbon. In glucopyranose, the anomeric carbon is C-1, and the prefix α- means the hydroxyl group is below the plane of the ring; the prefix β- means the hydroxyl group is above the plane of the ring. In fructofuranose, the anomeric carbon is C-2 (Stryer 1981).

The major sugars found in nectars and honeydews are mono-, di-, and trisaccharides. The monosaccharides glucose and fructose make up the majority of di- and trisaccharides. Along with sucrose, glucose and fructose are the major components in nectars and some honeydews. Sucrose is a disaccharide of glucose and fructose. The glucose is in an α-glucosidic linkage and the fructose is in a β-fructofuranosidic linkage. Maltose is a disaccharide of glucose in an α-(1,4)-glucosidic linkage.

Melezitose is a trisaccharide of glucose and sucrose. The additional glucose molecule is linked to the fructose moiety by an α-(1,3)-glucosidic linkage. Raffinose is a trisaccharide of galactose and sucrose. The galactose is in an α-(1,6)-galactosidic linkage to the glucose moiety of the sucrose.

Carbohydrates are generally absorbed as monosaccharides, so that disaccharides and polysaccharides must be broken down to their component monosaccharides before they can be absorbed (Chapman 1982). Carbohydrases are enzymes that catalyze the breakdown of various glycosides and oligosaccharides. Carbohydrases are classified according to the nature of the sugar moieties in the glycoside and the anomeric configuration of the glycosidic linkage hydrolyzed, but are usually named according to the type of bond hydrolyzed (Nisizawa and Hashimoto 1970). There are general carbohydrases such as an α-glucosidase that hydrolyze sucrose, maltose and trehalose, and there are more specific carbohydrases that hydrolyze a single substrate such as trehalose (Chapman 1982). Carbohydrases reported from various species of insects are α-glucosidases, β-glucosidases, α-galactosidases, β-galactosidases, and β-fructofuranosidases (McFarlane 1985).

CHAPTER III

MATERIALS AND METHODS

High Performance Thin Layer Chromatography (HPTLC)

HPTLC Method

The method used for detecting sugars by HPTLC was obtained from Richard D. Fell, Associate Professor of Entomology, at Virginia Polytechnic Institute and State University at Blacksburg, Virginia, in a letter prior to publication. Precoated Merck glass HPTLC Silica Gel 60 plates were prewashed in methanol and dried. Plates were then sprayed with a 0.1 M sodium bisulfite solution, dried, sprayed with a 10 mM citrate buffer solution, pH 4.8, dried, and activated by heating in a 100°C oven for one hour. The 10 mM citrate buffer solution was prepared using 90 mM Sigma Citrate Buffer solution, pH 4.8 at 25°C, (Sigma, St. Louis, MO) and diluted 1:9 in water. Prepared plates were stored in a desiccator.

Standard sugar solutions and diluted crop contents were spotted 1 cm from the bottom, 5 mm apart on plates with a Camag Nanomat II (Wrightsville, NC) using 1 μl disposable microcapillary tubes and allowed to completely dry before

development. The developing solution of acetonitrile/water (87:13) consisted of HPLC grade acetonitrile and deionized water from a Millipore Milli-Q System (Millipore, Bedford, MA) for a total volume of 30 ml. The developing chamber was a Camag twin trough chamber for 20 X 10 cm HPTLC plates and the developing solution was in one trough with a saturation pad and allowed to condition for 10 min before developing. Plates were developed four times in the same direction, running the solvent 8 cm from the origin, in the same solvent and tank, and drying between each development. The plates were dipped vertically in a ceric sulfate solution consisting of 0.1 N ceric sulfate diluted 1:10 in 15% sulfuric acid. The 0.1 N ceric sulfate in approximately 2 N sulfuric acid was purchased from American Scientific. After dipping, the plates were drained vertically for approximately one minute and the glass backing wiped dry. Plates were heated in an oven at 115°C for 15 minutes to char the sugars for visualization. Spots were identified by comparison to sugar standards run on the same plate.

Plates were scanned using an LKB Ultroscan XL Laser Densitometer (LKB, Broma, Sweden) interfaced with a microcomputer using GelScan XL software and data was stored on 5 1/4 inch (13.335 cm) diskettes. The densitometer used a helium-neon laser providing monochromatic light at 633 nm (line beam, 50 μm X 800 μm) for transmission scanning.

Validation Procedure

Before crop sugars were analyzed using the HPTLC method described above, standard laboratory concentrations of sugar solutions were prepared to optimize the HPTLC method for the separation of all sugars expected to be in the crops of horse flies. Sugars were purchased from Sigma Chemical Co. (St. Louis, MO). Solutions of the eight sugars raffinose, melezitose, trehalose, maltose, turanose, sucrose, glucose and fructose were prepared in concentrations of 0.25, 0.5, 1.0, 2.0, and 3.0 $\mu g/\mu l$ and served as the sugar standards. A five sugar standard containing melezitose, maltose, sucrose, glucose and fructose, and a seven sugar standard containing these five sugars plus raffinose and trehalose were prepared in the same concentration as the single sugar standards. All of these sugars occur naturally in nectar and honeydew, and therefore could be naturally occurring in the crops of horse flies that fed on them. The five and seven sugar standards were used to verify that the multiple sugars in combination would separate and be comparable to the individual standards run on the HPTLC plate.

Analysis of Natural Crop Sugars

Specimen Collection

H. lasiophthalma and T. abactor females were collected at the Cross Timbers Experimental Range in Payne County, Oklahoma, which is dominated by Postoak-Blackjack Forest

type vegetation interspersed with grassland (Ewing et al. 1984). Specimens were captured in two modified Stoneville Malaise traps (Roberts 1976) made of Lumite screen (Chicopee Manufacturing Co., Gainsville, FL)(Cooksey and Wright 1987). A 2 l plastic soft drink bottle fitted with two funnels was used as the killing chamber by securing a 6 X 2 cm piece of dichlorovos-impregnated resin in the bottle to quickly kill captured insects that were directed into the bottle by the first funnel (Fig 1). The second funnel directed fallen insects into a 3 cm tube that went to a 1 l polypropylene jar inside a styrofoam bucket containing ca. 4.3 kg of dry ice which froze specimens and acted as an attractant, as the sublimating CO_2 gas escaped through three 2 cm holes in the side of the bucket (Fig 2). Additional dry ice was added to the bucket during the later part of the day to keep specimens frozen. Specimens were collected hourly throughout the daylight hours, sorted to species, maintained on dry ice until the end of the day, and then stored at -40°C. Traps were operated once a week to collect H. lasiophthalma females from April 19 to May 8, 1987 and to collect T. abactor females from June 13 to August 12, 1987. Male specimens were collected in the morning while they were hovering (Szumlas 1988, Wilkerson et al. 1985), put in 35 ml plastic cups with paper lids, maintained on dry ice until the end of the day, and then stored at -40°C.

Fig 1. Killing chamber used on the modified Stoneville Malaise trap.

Fig 2. Configuration of the modified Stoneville Malaise trap used to freeze collected specimens.

Dissection Technique

Frozen specimens were individually thawed and dissected. The cuticle of abdominal tergum 2 was cut laterally and then each pleural membrane was cut caudally, stopping before the anus. Care was taken not to damage internal organs. The specimen was pinned off-center through the dorsum of the thorax in a paraffin filled dish, and the dorsum of the abdomen was pinned back, revealing the internal organs. The crop was removed intact by grasping only the crop duct and lifting the crop out. The crop was rinsed with deionized water and gently blotted to remove external fluid and placed on a glass microscope slide that had been wrapped with Parafilm M. The crop was opened and a known amount of crop fluid (0.1 - 0.5 μl) was drawn into a 0.5 μl Hamilton microsyringe and placed in a 1.5 ml micro centrifuge tube. The fluid was diluted 50X with 50% methanol (v:v) to stop enzyme activity and stored at -40°C until analyzed for sugars using HPTLC as described above. The 2.0 μg/μl concentration of the five sugar standard was used as a standard when analyzing crop contents.

Laboratory Feeding Studies

In the laboratory feeding studies, specimens were fed sucrose, maltose, or melezitose because they are the di- and trisaccharides reported present in nectar and honeydew.

Live specimens of H. lasiophthalma and T. abactor females were collected from cattle and H. lasiophthalma

males were collected in the morning while they were hovering. Specimens were taken to the laboratory, put in 550 ml paper cartons and maintained at 30±1°C and 55±5% RH, with a 16:8 (L:D) photoperiod. Ten specimens of the same sex were placed in each of eight cartons prepared for each of the three sugars for a total of 72 cartons. A nylon mesh screen covered the top of the carton and the bottom was replaced with a plastic petri dish containing filter paper. This arrangement allowed viewing and feeding of the specimens from the top and collecting of excrement in the disposable filter paper in the bottom. A cotton ball saturated with water was placed on the screen top of each carton.

Because horse flies were collected in nature, the age and nutritional state of the specimens were not known, other than the females were seeking a blood meal. Both T. abactor and H. lasiophthalma are anautogenous and mated prior to blood feeding. Most field collected specimens have some sugars in the crop when collected (Bosler and Hansens 1974, Leprince and Jolicoeur 1986, Leprince and Lewis 1986). To empty the crop of any natural sugars, specimens were provided with only water for 48 hrs and 24 hrs for female and male specimens respectively. After this period, dead flies were removed, and seven cartons of flies were provided a cotton ball saturated with a 5% (w:v) solution of either sucrose, maltose, or melezitose for two hours. For each group of flies, those in seven cartons were fed a particular

sugar, while those in the eighth carton served as a control and were maintained on water only. The control flies were used to confirm that the deprivation time was adequate to empty the crop of natural sugars. Each sugar was fed two hours and then replaced with deionized water. This time was designated 0 hrs after feeding and samples of flies were collected at 0, 2, 4, 6, 8, 12, and 24 hrs after feeding. At each designated time, the appropriate cartons of flies were stored at -40°C to stop metabolic processes. Controls were stored at 0 hrs. The crops were later dissected and the contents subjected to HPTLC analysis as described above to detect the sugars remaining in the crop.

Preparation of Salivary Glands to Determine Presence of Digestive Enzymes

Dissection of Salivary Glands

Live specimens of H. lasiophthalma males and females and T. abactor females were immobilized by chilling in a refrigerator. A specimen was place ventral side up in a depression in a paraffin filled dish and secured with a portion of rubber band stretched across the thorax and pinned to the paraffin. A straight pin was used to move the head apically to stretch the neck. The common salivary duct, which lies just below the transparent cuticle of the neck (West 1951), was grasped with fine forceps and slowly pulled apically to remove the salivary glands.

Incubation in Sugar Solutions

The common salivary duct and the two attached salivary glands were rinsed with deionized water. The salivary glands from an individual specimen were macerated with the head of an insect pin in 10 µl of a 5% solution of either sucrose, maltose, or melezitose in a 500 µl micro centrifuge tube. Homogenates of salivary glands and sugar were incubated for four hours at 30°C, after which 10 µl of 50% methanol was added and the mixture stored at -40°C. The 50% methanol was added to dilute the sugar mixture and to stop any enzyme activity. Incubation mixtures were later thawed and subjected to HPTLC analysis as described above.

Five individual salivary gland homogenates for H. lasiophthalma females, males, and T. abactor females were prepared in each of the three sugars for a total of 15 salivary gland homogenates in each sugar sucrose, maltose, and melezitose. Salivary glands of five T. abactor females were also incubated in raffinose.

Microassays with p-Nitrophenyl Substrates

Carbohydrases are specific for the linkage and sugar moiety. Commercially prepared p-nitrophenyl compounds were used to assay for specific enzymes. The p-nitrophenyl substrate consists of a sugar moiety bound to p-nitrophenol in a specific linkage, either alpha or beta. Incubation of the p-nitrophenyl substrate with the enzyme specific for the sugar moiety and linkage will under proper conditions

hydrolyze the substrate releasing p-nitrophenol which is yellow. A survey for specific carbohydrases can be performed by using p-nitrophenyl substrates with different linkages and different sugar moieties. Sucrose, maltose, and melezitose are hydrolyzed by α-glucosidases. Sucrose is also hydrolyzed by β-frucotofuranosidases. Raffinose is hydrolyzed by α-galactosidases and β-fructofuranosidases.

Enzyme microassays using p-nitrophenyl substrates for qualitative determination of enzymatic activity followed the procedure as described by Young et al. (1987). Enzyme reactions were carried out in flat-bottomed 96-well microtiter plates. Salivary gland homogenates for use with the p-nitrophenyl substrates were prepared with glands from male and female T. abactor. Salivary glands of ten flies were dissected as above and were rinsed with 50 mM Tris buffer pH 7.5. Salivary glands were then homogenized in 50 µl of ice cold Tris buffer using a 0.5 ml tissue grinder with a Teflon pestle and the pestle rinsed with 50 µl of ice cold buffer for a total volume of 100 µl of enzyme sample. Samples were centrifuged for one minute at 7°C and the supernate transferred to a clean 500 µl micro centrifuge tube and kept on ice.

Assay substrates were p-nitrophenyl-α-D-glucopyranoside (PNP-α-glc), p-nitrophenyl-β-D-glucopyranoside (PNP-β-glc), p-nitrophenyl-α-D-galactopyranoside (PNP-α-gal), and p-nitrophenyl-β-D-galactopyranoside (PNP-β-gal) (Sigma Chemical Co., St.

Louis, MO). Aqueous 5 mM solutions of each substrate were kept frozen in 500 μl aliquots and thawed only once before use. The p-nitrophenyl substrates and refrigerated 0.5 M sodium acetate solution, pH 5, were mixed 1:1 immediately before the assay to give the final substrate solution. The reaction mixture consisted of 10 μl of enzyme sample and 125 μl of substrate solution per well. Samples were incubated at 24°C for 1, 4, 8, 12, 16, 20, 24, and 48 hours. Reactions were stopped by adding 100 μl of ice cold 0.1 M NaOH to each well. Absorbance of the solution was then determined using a microplate reader at 405 nm.

CHAPTER IV

RESULTS AND DISCUSSION

HPTLC of Sugar Standards

The HPTLC method allowed for highly reproducible separation of the mono-, di-, and trisaccharides at concentration from 0.25 to 3.0 μg/μl, spotting 1 μl (Fig 3). Silica Gel does not give satisfactory separation of sugars unless it is impregnated with inorganic salts (Ghebregzabher et al. 1976). In the system described by Fell (pers. comm.) and used in this study, the 0.1 M sodium bisulfite provided the needed inorganic salt. Fell's method was modified by changing the solvent ratio from 85:15 to 87:13 acetonitrile/water. Since the 85:15 ratio failed to resolve glucose and fructose. To get greater separation of glucose and fructose for transmission densitometer scanning the plate was developed four times instead of three. The resolution obtained with the five sugar standard of melezitose, maltose, sucrose, glucose, and fructose at 2.0 μg/μl, spotting 1 μl, provided good separation of peaks using the densitometer (Fig 4). The densitometer detected 0.5 μg/μl and higher concentrations of sugars. The 2.0 μg/μl concentration of the five sugar standard was chosen to run with the crop samples because it produced dark spots

Fig 3. High performance thin layer chromatography plate showing the separation of sugars using the system described in text: numbers 1), 5), and 8) are the five sugar standard, 2 μg/μl, 1 μl spotted; 2) is the seven sugar standard, 1 μg/μl, 1 μl spotted; 3), 6), and 7) are the seven sugar standard, 1 μg/μl, 0.5 μl spotted; 4) is the seven sugar standard, 0.5 μg/μl, 0.5 μl spotted. The sugars in the seven sugar standard appear on the plate in ascending order: raffinose, melezitose, trehalose, maltose, sucrose, glucose, and fructose.

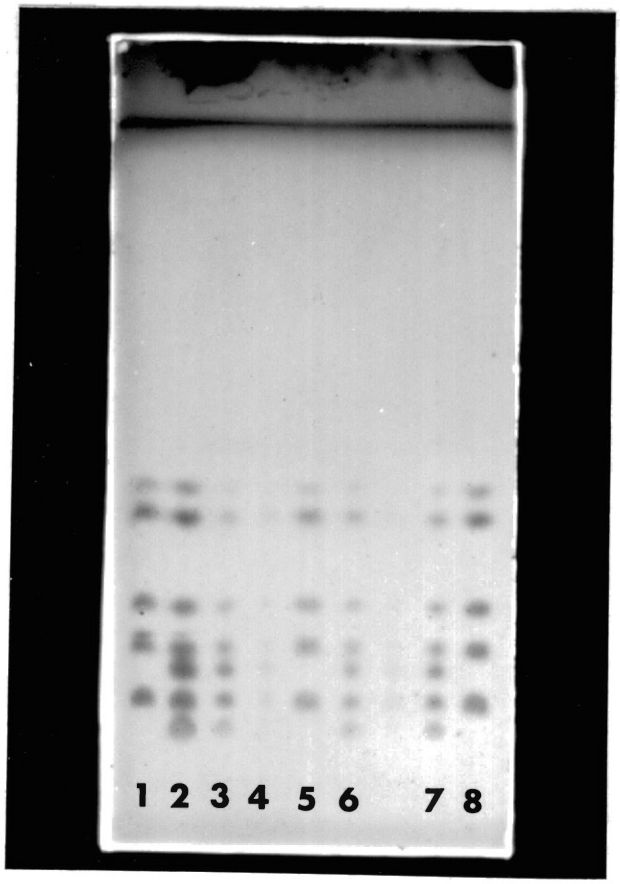
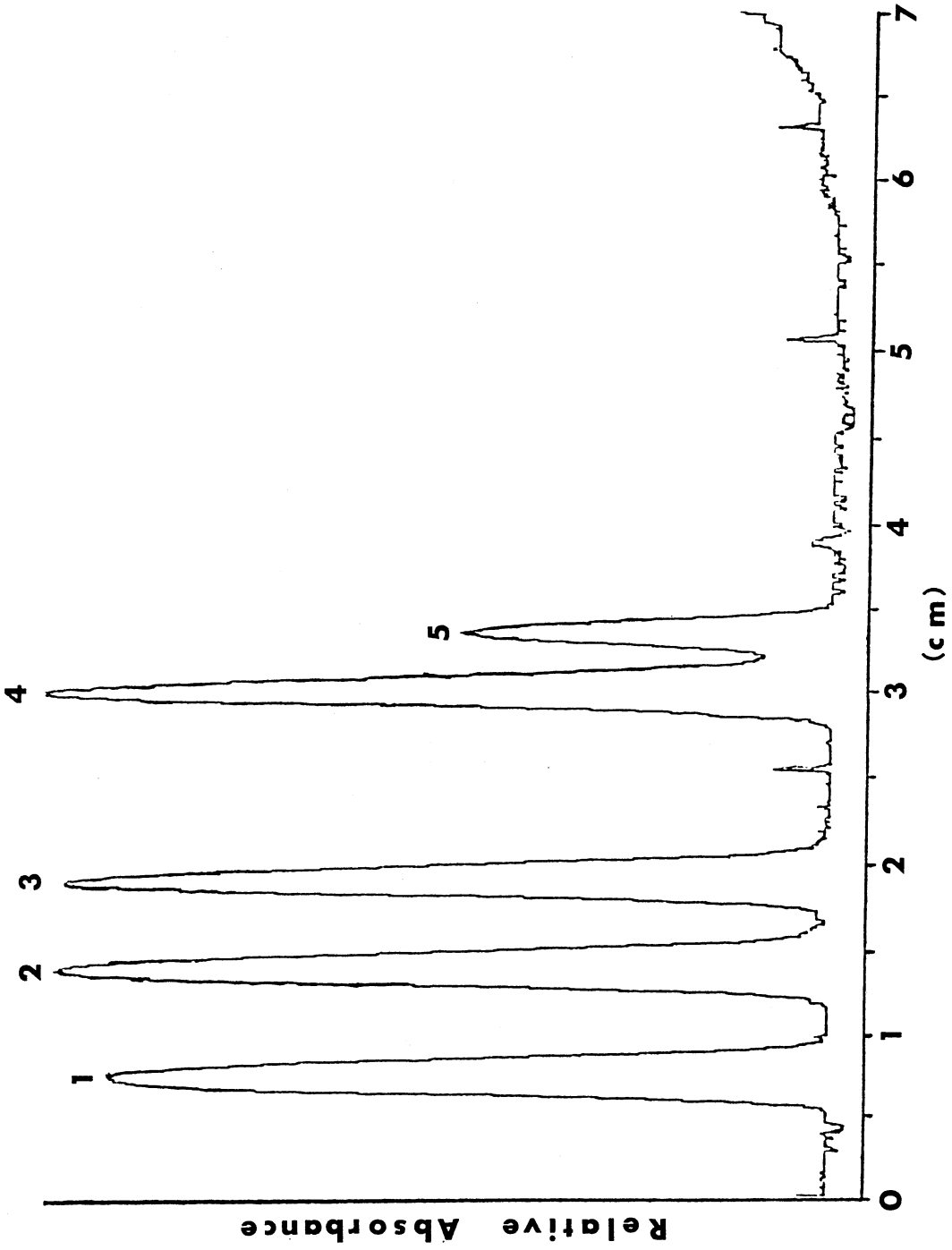

1 2 3 4 5 6 7 8

Fig 4. Densitogram of the five sugar standard number 8) in Fig 3. Numbered peaks are 1) melezitose, 2) maltose, 3) sucrose, 4)glucose, 5) fructose. Origin is at O and solvent front was 8 cm from origin.


Relative Absorbance
1
2
3
4
5
0
1
2
3
4
5
6
7
(cm)

without tailing. The resolution between the five sugars was great enough to obtain good separation of densitometer peaks while allowing space to detect sugar peaks that would occur between them. For example, trehalose occurs between melezitose and maltose, and turanose between maltose and sucrose.

The detection of insect carbohydrates has the inherent difficulties of small volume, small amount of sugar, and possible contaminates. These difficulties can be overcome by the use of high performance thin layer chromatography. The HPTLC method for determination of mono-, di-, and trisaccharides using acetonitrile/water 87:13 is a fast and simple method compared to high pressure liquid chromatography or gas-liquid chromatography that requires derivitization. With HPTLC a large number of samples can be run at one time. On 20 X 10 cm plates, 39 positions are available for samples, and the development of plates four times takes approximately one hour. The HPTLC plates used in this study were of precoated glass plates which provides consistency, Silica Gel particles 5 μm in diameter instead of 12 μm particles used in common TLC plates, closer grain distribution, and 0.2 mm layer thickness instead of 0.25 mm. With the use of these more uniform "high performance" plates, sugar concentrations down to 0.25 μg/μl, spotting 1 μl, were detected.

The LKB XL laser densitometer scans in only the transmission mode which requires the beam to travel through

the glass plate, silica absorbent, and charred sugar spot before recording. The sugars were approximately linear from 0.25 to 2.0 μg/μl and lost linearity at 3.0 μg/μl as described by Fell (pers. comm.).

Natural Crop Contents

A total of 88 and 16 H. lasiophthalma females and males respectively, were dissected and 92 and 100% had fluid in the crop. A total of 203 and 18 T. abactor females and males respectively, were dissected and 32 and 56% had fluid in the crop. Crop contents were analyzed from specimens collected at all times of the day and throughout the season and there was no detectable patterns associated with time of day or seasons for any sugar combination.

The HPTLC of natural crop contents of 75 T. abactor and 97 H. lasiophthalma, total of 172 specimens, indicated the presence of one or more of the sugars fructose, glucose, sucrose, maltose, and melezitose in each crop (Table I). Trehalose was detected in several samples but was considered a contamination from the hemolymph. Three of 65 and 8 of 81 T. abactor and H. lasiophthalma females respectively had sucrose as the only sugar detected in the crop. Melezitose, glucose and fructose was detected in 33.85 and 20.0% of the T. abactor crops, females and males respectively. None of the H. lasiophthalma had this combination of sugars. The percentage of each sugar detected in the crops was 84.8% (146/172) fructose, 93.0% (160/172) glucose, 54.1% (93/172)

TABLE I

SUGARS DETECTED IN THE CROPS OF TWO SPECIES OF HORSE FLIES COLLECTED UNDER FIELD CONDITIONS

Sugars in Crop[a]					*Tabanus abactor*				*Hybomitra lasiophthalma*				Total	
					Female		Male		Female		Male			
F	G	S	Ma	Me	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
+	+				15	(23.1)	6	(60.0)	11	(13.6)	3	(18.6)	35	(20.4)
+	+	+			3	(4.6)	-		26	(32.1)	1	(6.2)	30	(17.4)
	+	+			-		-		2	(2.5)	-		2	(1.2)
		+			3	(4.6)	-		8	(9.9)	-		11	(6.4)
+	+	+	+		2	(3.1)	-		8	(9.9)	-		10	(5.8)
+	+		+		-		1	(10.0)	-		1	(6.2)	2	(1.2)
+	+		+	+	7	(10.8)	-		2	(2.5)	9	(56.2)	18	(10.5)
+	+			+	22	(33.8)	2	(20.0)	-		-		24	(14.0)
+	+	+		+	5	(7.7)	-		14	(17.3)	-		19	(11.0)
+	+	+	+	+	7	(10.8)	1	(10.0)	8	(9.9)	2	(12.5)	18	(10.5)
	+	+	+	+	-		-		1	(1.2)	-		1	(0.6)
	+	+		+	1	(1.5)	-		-		-		1	(0.6)
		+	+	+	-		-		1	(1.2)	-		1	(0.6)
				Total	65	(100)	10	(100)	81	(100)	16	(100)	172	(100)

[a] F = fructose, G = glucose, S = sucrose, Ma = maltose, Me = melezitose.
(+) indicates the presence of sugar, not the amount of sugar, detected.

sucrose, 29.1% (50/172) maltose and 47.7% (82/172) melezitose.

Magnarelli and Anderson (1981) used TLC and reported 94% of H. lasiophthalma females contained fructose or sucrose. Of those 94%, 23.8% had glucose and fructose, and 70.3% had sucrose, glucose and fructose. Their TLC method used the entire specimen, so their results are of total specimens examined. Of the total specimens examined in the study, 12.5% of H. lasiophthalma females contained the combination of glucose and fructose, and 63.6% had sucrose, glucose and fructose. The total in the present study was 76.1% sugar-positive according to the definition of Magnarelli and Anderson (1981). By contrast, T. abactor had a very low percentage, 32% of 203, of specimens having fluid in the crop compared to H. lasiophthalma with 92% of 88 specimens. Glucose and fructose was detected in 21.7% and sucrose, glucose and fructose was detected in 9.8% of total specimens examined.

Compared to 53 to 94% anthrone positive and TLC positive specimens in other tabanid species (Magnarelli and Anderson 1977, 1981, Magnarelli et al. 1979, Kniepert 1980, Leprince and Jolicoeur 1986, Leprince and Lewis 1986), the prevalence of sugar positive T. abactor was low. The use of traps for the collection of specimens in this study was based on the traps measuring the same daily activity patterns as observed on a cow (Hollander and Wright 1980b). The energy used by specimens collected in traps was not

taken into account in this study. The number of T. abactor that did not have fluid in the crop could be caused by a large amount of energy being expended while being collected in the trap.

All sugars detected in the crops of field collected H. lasiophthalma and T. abactor (Table I) have been reported in nectars (Baker and Baker 1982) and in homopteran honeydews (Auclair 1963). The presence of melezitose in 47.7% of the total number of crop contents examined and maltose in 29.1% was similar to what Magnarelli and Anderson (1981) reported in Connecticut, USA, with TLC on 16 species of tabanids, including H. lasiophthalma. They reported these sugars in trace amounts, while in the present study, melezitose was a primary component in several of the crops examined.

Laboratory Feeding Studies

The crops of all 43 control specimens that survived the starvation period were empty, confirming that the starvation period had depleted any natural sugars contained in the crop. The experimental design for each sample period for each sugar was to have at least five or more specimens alive at the time of feeding. Even after providing the cages with sugar solution several flies died, possibly because they did not find the sugar solution. Specimens from each sample period were dissected until five crops containing fluid were obtained or until all specimens alive at the time of freezing had been dissected. The crops of some specimens

were empty, but several of these specimens, as well as several that had fluid in the crop, had large amounts of fluid in the ventriculus and pylorus. This was most likely a result of extreme starvation, causing the fly to direct carbohydrates to the midgut for immediate use to sustain life. Fluid was present in the crops of 25% (44/176) of the T. abactor and 52.3% (57/109) of the H. lasiophthalma for a total of 35% (101/285) of the specimens dissected.

Only 42 of 66 (66.7%) H. lasiophthalma fed sucrose had fluid in the crop. Nine of 11 and five of six specimens (over 80%) did not have sucrose in the crop at 0 and 2 hrs after feeding, respectively, Table II. Only one of the 25 remaining specimens had sucrose in the crop, that being eight hours after feeding, Table II. These data indicate that hydrolysis of sucrose in the crop must occur rapidly, as 82% of those females fed sucrose for 2 hours had only glucose and fructose in the crop at the 0 hr sample period. Only six of 27 (22.2%) female and one of seven male H. lasiophthalma fed on maltose had fluid in the crop and all but one female, from the 0 hr period, had hydrolyzed the maltose in the crop to glucose by 2 hours after feeding, Table III. Fifteen of 29 (36.5%) female and one of four male H. lasiophthalma fed melezitose had fluid in the crop and all but four females had melezitose in the crop as well as its component sugars of glucose and fructose, Table IV. The four specimens not having melezitose were from 0, 2, 12 and 24 hours after feeding, while four other specimens from

TABLE II

SUGARS DETECTED IN THE CROPS OF TWO SPECIES OF HORSE FLIES FROM 0-24 HOURS AFTER FEEDING ON A 5% SUCROSE SOLUTION.

Species Type	Sample Hour	Number of Flies Dissected per Sample	Number of Flies With Fluid In Crop	Sugar(s) Detected In Crop			
				Sucrose	Glucose	Fructose	No.
H. lasiophthalma							
Females	0	20	11	+	+	+	2
				-	+	+	9
	2	8	6	+	+	+	1
				-	+	+	5
	4	11	10	-	+	+	10
	8	9	3	+	+	+	1
				-	+	+	2
	12	11	7	-	+	+	7
	24	7	5	-	+	+	5
Males	0	11	1	+	-	-	1
T. abactor							
Females	0	8	5	+	-	-	4
				+	+	+	1
	2	8	5	+	+	+	5
	4	9	6	+	+	+	3
				-	+	+	3
	6	8	5	+	-	-	1
				+	+	+	1
				-	+	+	3

TABLE III

SUGARS DETECTED IN THE CROPS OF TWO SPECIES OF HORSE FLIES FROM 0-24 HOURS AFTER FEEDING ON A 5% MALTOSE SOLUTION.

Species Type	Sample Hour	Number of Flies Dissected per Sample	Number of Flies With Fluid In Crop	Sugar(s) Detected In Crop		
				Maltose	Glucose	No.
H. lasiophthalma						
Females	0	5	1	+	-	1
	2	4	1	-	+	1
	4	5	2	-	+	2
	8	5	1	-	+	1
	12	4	1	-	+	1
	24	4	0			0
Males	4	7	1	-	+	1
T. abactor						
Females	0	8	6	+	-	1
				+	+	4
				-	+	1
	2	10	5	+	-	1
				-	+	4
	4	7	4	+	+	3
				-	+	1
	6	6	6	-	+	6

TABLE IV

SUGARS DETECTED IN THE CROPS OF TWO SPECIES OF HORSE FLIES FROM 0-24 HOURS AFTER FEEDING ON A 5% MELEZITOSE SOLUTION

Species Type	Sample Hour	Number of Flies Dissected per Sample	Number of Flies With Fluid In Crop	Sugar(s) Detected In Crop			
				Melezitose	Glucose	Fructose	No.
H. lasiophthalma							
Females	0	5	2	+	-	-	1
				-	+	+	1
	2	4	2	+	+	+	1
				-	+	+	1
	4	6	2	+	-	-	2
				+	+	+	1
	8	5	4	+	+	+	4
	12	5	2	+	-	-	1
				-	+	+	1
	24	4	3	+	-	-	1
				+	+	+	1
				-	+	+	1
Males	8	4	1	+	+	+	1
T. abactor							
Females	0	10	4	+	+	+	4
	2	9	6	+	+	+	6
	4	6	3	+	+	+	2
				-	+	+	1
	6	10	3	+	+	+	3
	8	19	3	-	+	+	3
	12	9	4	-	+	+	4

0, 4, 12, and 24 hours had only glucose and fructose in the crop. Apparently, H. lasiophthalma does digest melezitose in the crop but not as rapidly as they did sucrose and maltose.

Twenty one of 33 (63%) T. abactor fed sucrose had fluid in the crop. Only three of the six specimens from the 4 hr period and three of five specimens from the 6 hr period for a total of six of 21 (28.6%) did not have sucrose in the crop, Table II. Twenty one of 31 (67.7%) specimens fed maltose had fluid in the crop. Five of six, one of five and three of four specimens had maltose in the crop at 0, 2, and 4 hr periods after feeding, Table III. All six of those specimens from the 6 hr period had hydrolyzed maltose to glucose. Twenty three of 63 (36.5%) T. abactor fed on melezitose had fluid in the crop. Only one of 16 specimens from the 0, 2, 4, and 6 hr periods did not have melezitose in the crop, while all specimens from the 8 and 12 hour periods had hydrolyzed the melezitose to glucose and fructose, Table IV. All 42 of the crops of specimens fed raffinose were empty.

Sucrose is hydrolyzed by an α-glucosidase or a β-fructofuranosidase. The hydrolysis of sucrose in the crop indicated that either an α-glucosidase or a β-fructofuranosidase that acts on sucrose was introduced into the crop. Maltose is hydrolyzed by an α-glucosidase. The hydrolysis of maltose in the crop indicated that an α-glucosidase that acts on maltose was introduced into the

crop. Melezitose is hydrolyzed by an α-glucosidase. The hydrolysis of melezitose in the crop indicated that an α-glucosidase for melezitose was introduced into the crop.

Hansen Bay (1978a) reported the fluid in the crop of laboratory fed C. erythrocephala is a mixture of saliva and sugar. The hydrolytic activity of C. erythrocephala saliva includes α-glucosidic, α-galactosidic, and amylase activities, all of which were observed in the crop. Hansen Bay (1978a) used additional substrates that were not used in this study to determine these hydrolytic activities. All of the di- and trisaccharides fed to H. lasiophthalma or T. abactor were hydrolyzed in the crop. This suggests there is an α-glucosidase and possible a β-fructofuranosidase in the crop with the sugar meal. The failure of any fluid to be found in the crops of specimens fed raffinose precluded the determination of an α-galactosidase or a β-fructofuranosidase.

Both species had a much higher percentage of sucrose and of melezitose in the crop compared to those specimens with maltose in the crop. This might reflect the feeding preference of these species for specific sugars. However, Friend and Stoffolano (1989) report that over 90% of the 20 T. nigrovittatus fed a 0.3 M sucrose solution directed it to the midgut or the midgut and the crop. In contrast, 1 M sucrose was directed to the crop in 70% of 20 specimens (Stoffolano 1983). In their study of the destination of water, ATP in 0.15 M NaCl, 1 M sucrose and 0.3 M sucrose fed

to T. nigrovittatus, Friend and Stoffolano (1989) conclude that the osmotic pressure of the solution, not the species of molecule, affects or controls diet destination. The 0.3 M sucrose solution was treated by the flies the same as the blood mimic of ATP in 0.15 M NaCl which has an osmolarity of 0.3. The molar concentration of the 5% sugar solution fed to H. lasiophthalma and T. abactor were: sucrose 146 mM , maltose 146 mM, melezitose 99 mM, and raffinose 99 mM. Investigations into the affects of osmotic pressure on the destination of sugar meals in T. abactor and H. lasiophthalma and on feeding preferences need to be conducted before further speculation can be made.

Salivary Gland Homogenates

Incubation in Sugars

Three of four, five of five and three of five of the salivary gland homogenates from H. lasiophthalma females, males and T. abactor females hydrolyzed sucrose, Table V. Nine of these 11 homogenates completely hydrolyzed sucrose.

All five of the salivary gland homogenates from H. lasiophthalma females completely hydrolyzed maltose and all five of the H. lasiophthalma males salivary gland homogenates partially hydrolyzed maltose, Table V. Only one of four salivary gland homogenates from female T. abactor partially hydrolyzed maltose, Table V.

The salivary gland homogenates from only one of 15 specimens, male H. lasiophthalma, hydrolyzed melezitose,

TABLE V

SUGARS DETECTED IN HOMOGENATES OF THE SALIVARY GLANDS OF TWO SPECIES OF HORSE FLIES IN 5% SOLUTIONS OF SUCROSE, MALTOSE, AND MELEZITOSE AFTER A FOUR HOUR INCUBATION PERIOD [a]

	Incubation Sugar											
	Sucrose				Maltose			Melezitose				
Species	Sugar(s) After Incubation [b]											
Type	S	G	F	No.	Ma	G	No.	Me	G	F	No.	
Control	+	-	-	1	+	-	1	+	-	-	1	
H. lasiophthalma												
Females	+	-	-	1	-	+	5	+	-	-	5	
	-	+	+	4								
Males	+	+	+	3	+	+	5	+	-	-	4	
	-	+	+	2				-	+	+	1	
T. abactor												
Females	+	-	-	2	+	-	4	+	-	-	5	
	-	+	+	3	+	+	1					

[a] n = 5 for each incubation sugar and species type. Control n = 1.

[b] S = sucrose, G = glucose, F = fructose, Ma = maltose, Me = melezitose.

Table V, which was quite different from the digestion of melezitose in the crops of live specimens. No salivary gland homogenates of T. abactor hydrolyzed raffinose after the four hour incubation period.

The hydrolysis of sucrose and maltose in the salivary gland homogenates indicates the presents of an α-glucosidase and possibly a β-fructofuranosidase. However, the raffinose was not hydrolyzed by the salivary gland homogenates of T. abactor females which indicates the absence of a β-fructofuranosidase or an α-galactosidase. The absence of a β-fructofuranosidase agrees with the melezitose not being hydrolyzed in the salivary gland homogenates. The pH of the homogenate was not known and other chemical and physical factors that would affect the enzymes in the salivary glands were not investigated. This might explain why melezitose was hydrolyzed in the crop but not in the salivary gland homogenates, unless the enzyme came from another source than the salivary glands.

Microassays with p-Nitrophenyl Substrates

The microassays using p-nitrophenyl substrates were used to help clarify what types of carbohydrases were in the salivary glands. The PNP-β-glc, PNP-α-gal, and PNP-β-gal were not acted on by the salivary gland homogenates with incubation times up to 48 hours. These results indicate that there is not a β-glucosidase, an α-galactosidase, or a β-galactosidase in the salivary gland homogenates of T.

abactor females or males. The PNP-α-glc was hydrolysed after 1 hour of incubation producing a yellow solution. The yellow color increased with extended incubation time. Homogenates of ten salivary glands of T. abactor females incubated with PNP-α-glc for 6 hours produced 28 μmoles of p-nitrophenol. Homogenates of ten salivary glands of T. abactor males incubated with PNP-α-glc for 12 hours produced 70 μmoles of p-nitrophenol. These results indicate the presents of an α-glucosidase in both female and male T. abactor salivary gland homogenates.

The positive results of an α-glucosidase in the salivary glands supports the results of the feeding study and the study incubating salivary gland homogenates with sugars. The absence of an α-galactosidase supports the results of the incubation of the salivary glands in raffinose.

In some preliminary investigations, a sucrose solution with phenol red was fed to five female T. abactor. The labellum of these specimens was then stimulated by a crystal of sucrose and a clear fluid, thought to be saliva, was extruded from the mouthparts. From one specimen, 1 μl of fluid was collected, and less than was 1 μl was collected from the other specimens. This fluid contained a PNP-α-glucosidase.

CHAPTER V

SUMMARY AND CONCLUSIONS

In order to achieve objectives one and two, the third objective of verifying a HPTLC method for the analysis of sugars had to be accomplished. The HPTLC method of Fell was modified from a ratio of 85:15 acetonitrile/water to one of 87:13 and the plates were developed four time instead of three. This method provided adequate separation of raffinose, melezitose, trehalose, maltose, sucrose, glucose and fructose.

The crop contents of field collected specimens of T. abactor and H. lasiophthalma were analyzed for sugars with the HPTLC method. Specimens of 104 T. abactor and 97 H. lasiophthalma were dissected and fluid was found in the crop of 75 T. abactor and 97 H. lasiophthalma. Various combinations of melezitose, maltose, sucrose, glucose, and fructose were detected in the 172 crop contents analyzed. All of these sugars have been reported in nectars and homopteran honeydews. Before attempts were made to associate the sugars in the crop with possible sugar sources in the field, the second objective to determine if sugars were being hydrolyzed in the crop, needed to be addressed.

Sugars fed to specimens of both species were hydrolyzed

in the crop, within two to four hours of feeding on 5% sucrose, maltose, or melezitose. These hydrolysis products detected in the crop indicated the probability that hydrolytic enzymes from the salivary glands or the midgut were present in the crop. Assuming that the horse fly, like the blow fly, regurgitates from the crop and not the midgut, only the potential enzyme activity from the salivary glands was investigated, but the possibility of the midgut as a source of enzymes needs to be investigated. The hydrolysis of sucrose and maltose when incubated with homogenized salivary glands, indicated that the salivary glands were the probable source of the hydrolytic enzyme. An α-glucosidase was detected in the salivary gland homogenates assayed with p-nitrophenyl-α-glucoside. No β-glucosidic, α-galactosidic, or β-galactosidic activity was detected with the incubations of salivary gland homogenates or the p-nitrophenyl substrate assays. If the α-glucosidase was in the saliva and goes into the crop with the sugar meal, hydrolysis of sucrose, maltose, and melezitose in the crop would occur. Because sugars were being hydrolyzed in the crop, the natural crop sugars could not be used as an accurate indicator of the source of the natural sugars.

Because of the similarity of the digestive system of the horse fly and blow fly, the detailed research of sugar digestion of the blow fly was used to facilitate this research of the horse fly. This study was preliminary, but the results revealed an aspect of the horse fly that had

until now not been investigated. The presence of an α-glucosidase in the salivary glands, if secreted in the saliva and taken into the crop with a sugar meal, indicates a more intricate system of feeding and digestion in the horse fly than had previously been known.

There were many questions for further research revealed by this study. The first question is whether the α-glucosidase in the salivary glands is in the saliva? If it is in the saliva, then what stimulates salivation? Also, the pH optimum and the substrate specificity of the salivary enzyme needs to be determined. An α-glucosidase was detected in the salivary glands, but is there a β-fructofuranosidase or other enzymes that were not detected because of improper pH, substrate specificity, or the presence of inhibitors? Additionally, the pH of the saliva and of the various regions of the alimentary canal need to be determined to understand where the enzymes are active. The possibility of other sources of enzymes was not investigated in the study. Does the crop have enzymes and do any enzymes from the midgut get into the crop? To answer these questions, the source of regurgitate will have to be determined in addition to determining what enzymes are present in the midgut.

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VITA

Kenneth Lynn Hoppe

Candidate for the Degree of

Master of Science

Thesis: NATURAL SUGARS DETECTED IN THE CROPS OF TWO SPECIES OF HORSE FLIES (DIPTERA: TABANIDAE) AND THE HYDROLYSIS OF SUGARS IN THE CROP AND IN SALIVARY GLAND HOMOGENATES

Major Field: Entomology

Biographical:

Personal Data: Born in Lawton, Oklahoma, May 2, 1960, the son of James E. and Thelma Jo Hoppe.

Education: Graduated from Eisenhower Senior High School, Lawton, Oklahoma, in 1978; received Bachelor of Science Degree in Biology from Oral Roberts University, Tulsa, Oklahoma in May, 1983; completed requirements for the Master of Science Degree at Oklahoma State University in July, 1989.

Professional Experience: Graduate Research Assistant, Department of Entomology, Oklahoma State University, Stillwater, Oklahoma, January, 1987, to July, 1989.

Societies: American Institute of Biological Sciences, American Mosquito Control Association, Entomological Society of America, Society for Vector Ecology.