# FACTORS AFFECTING CUTICULAR HYDROCARBON ANALYSIS FOR IDENTIFICATION OF FIELD POPULATIONS OF *TABANUS MULARIS* STONE (DIPTERA: TABANIDAE) IN OKLAHOMA

By

ROBERT A. MIHALOVICH

Bachelor of Arts

Washington and Jefferson College

Washington, Pennsylvania

1993

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 1996

FACTORS AFFECTING CUTICULAR HYDROCARBON ANALYSIS FOR IDENTIFICATION OF FIELD POPULATIONS OF *TABANUS MULARIS* STONE (DIPTERA: TABANIDAE) IN OKLAHOMA

Thesis Approved:

Russell W Wright
Thesis Adviser

Jack W. Dillwith

Richard Berberet

Thomas C. Collins
Dean of the Graduate College

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Dr. Russell Wright and the Department of Entomology for their generous support throughout the course of this project. I also thank Dr. Richard Berberet for serving as a member of my committee.

Very special thanks goes to Dr. Jack Dillwith for his invaluable assistance and to Dr. William Warde and the late Dr. W. Scott Fargo for helping me with the statistical analysis.

I also express my appreciation to Lisa Cobum and Richard Grantham for all of their valuable time and assistance. A very special word of thanks goes to Sarah McLean for her thoughtfulness, support and data processing ability. Most of all, my deepest appreciation goes to my parents Robert and Barbara and my family and friends who supported me throughout this study.

I dedicate this work to the memory of my grandfather Slim, and my uncle Nick who were always an inspiration to me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

## INTRODUCTION

The Brachyceran family Tabanidae (Diptera) consists of about 3500 species of medium to large biting flies, and contains some of the largest blood feeding insects on earth (LeHane 1991). Approximately 350 species of horse and deer flies occur in North America (Borror *et al.* 1989), and economic loss in livestock production due to horse flies exceeds 40 million dollars annually (Steelman 1976, Scholl *et al.* 1985). Haematophagous females in large numbers can cause economic damage to man and livestock through blood loss, annoyance, and disease transmission (Granett *et al.* 1957, Hollander and Wright 1980a, Perich *et al.* 1986). Some of the diseases transmitted by horse flies include anaplasmosis, anthrax, hog cholera virus, equine infectious anemia virus and Western equine encephalitis virus (Krinsky 1976).

The greenhead horse fly, *Tabanus mularis* Stone, is the second most abundant species of horse fly in north central Oklahoma (Wright *et al.* 1984). This species aggressively attacks cattle and belongs to what is perceived to be a "complex" of species which are difficult to distinguish morphologically. Identification procedures that complement the use of morphological characteristics involve the use of molecular markers. One such technique involves analyzing the cuticular hydrocarbons of insects to identify them to genus and species.

Cuticular hydrocarbon analysis was first proposed by as a taxonomic tool to aid in the identification of insects (Jackson and Blomquist ; Lockey (1976). Since that time, numerous authors have used this type of analysis to differentiate between closely related groups of medically important Diptera (Phillips et al. 1988). There is a need to determine if the horse flies identified as *T. mularis* in Oklahoma constitute a single group or belong to several strains of flies within the species. A preliminary study was designed to determine whether the analysis of

cuticular hydrocarbons could be applied to differentiate between *T. mularis* and the closely related species *Tabanus quinquevittatus* Wiedemann. In the course of this study the two species were successfully separated based on their hydrocarbon profiles. However, there was unexplained variability of the hydrocarbon profiles within the *T. mularis* species which appeared to be associated with the conditions under which the specimens were stored. It became evident that additional experiments were required to explain the variability of the results and to validate the procedures used in conjunction with cuticular hydrocarbon analysis.

To accomplish the overall goal of determining the utility of cuticular hydrocarbon analysis for identifying *T. mularis*, the following objectives were proposed:

1. Determine the effects of different collection and storage conditions on the amount and composition of extractable cuticular hydrocarbons from field collected *T. mularis*.
2. Determine if there were differences in the amounts and composition of extractable cuticular hydrocarbons from specimens collected throughout a season.
3. Determine the composition of extractable cuticular hydrocarbons from specimens collected in different geographic populations.

# CHAPTER II

## LITERATURE REVIEW

### *Tabanus mularis* Complex

In 1986,Wright *et al.* reported 64 species of Tabanidae in Oklahoma. Six of these species constituted 97.7% of the collections in northcentral Oklahoma during an earlier study (Wright *et al.* 1984). *Tabanus mularis* was identified as the second most abundant pest species in this study, occurring from 25 May- 20 September. *Tabanus mularis* is found in the central and southeastern U.S., with its range extending from the panhandle of Florida, northward to Maryland and westward to Missouri and eastern Texas (Pechuman *et al.* 1983). It occurs in most counties of Oklahoma east of the panhandle. Its seasonal activity in Oklahoma ranges from 29 May- 23 October (Wright et. al. 1984). The diurnal period of activity for *T. mularis* in Oklahoma is between about 9 AM and 6 PM CDT (Hollander and Wright 1980b).

Little is known about the biology of this fly, and only recently has the larval anatomy been described (Goodwin 1994). This species belongs to a "complex" of closely related horse flies commonly referred to as the "greenheads" (Richard Roberts, Florida State University, Personal Communication). The greenhead complex is generally considered to include five other Nearctic species; *Tabanus conterminus* Walker, *Tabanus fulvilineis* Philip, *Tabanus fuscicostatus* Hine, *Tabanus nigrovittatus* Macquart and *Tabanus quinquevittatus* Wiedemann. The possibility exists for these flies to represent two complexes, one a coastal group in which the larvae inhabit salt marshes, and another ranging from the coast to the interior of the U.S. where the larvae occur in a terrestrial habitat (Goodwin, 1994). Other species which have been placed in this group or as close relatives include *Tabanus eadsi* Philip (Tidwell 1973), *Tabanus fulvilineis* Philip (Goodwin 1994), *Tabanus lineola hinellus* Philip (Tidwell 1973; Freeman 1987), and *Tabanus texanus* Hine (Thompson 1973; Tidwell 1973). Sibling species of *T. mularis* in Oklahoma which share its geographic distribution include *Tabanus quinquevittatus* Wiedemann, and *Tabanus fuscicostatus* Hine. This latter species was reported in Oklahoma by Howell and Schomberg

(1955), but none were collected by Wright *et al.* (1986). *Tabanus fuscicostatus* also occurs however in Texas and in Louisiana (Tidwell 1973). *Tabanus mularis* is similar to darker forms of *T. nigrovittatus* Macquart, and some specimens are difficult to separate from *T. fuscicostatus* (Tidwell 1973).

The taxonomic problems that this group of flies presents has been well documented. Most of the characters now used to differentiate specimens are confined to the head and appendages (Pechuman 1981). Additionally, several members of this complex are identified to species or subspecies taxons based on color and pilosity, particularly on the labial palps and the pleural areas of the thorax ( Tidwell 1973, Pechuman *et al.* 1983). In many specimens this coloration is very difficult to distinguish, particularly if the specimens have been damaged or pinned for long periods of time, resulting in discoloration. For instance, *T. mularis* is supposed to have a whitish color on the hairs of the labial palps and pleuron, while *T. quinquevittatus* has a creamy yellow color (Pechuman *et al.* 1983). This determination is not always easily made.

Tabanids in this study are classified according to the phylogenetic arrangement described by Philip (1965) and Wright *et al.* (1986).

| | |
|---|---|
| Order | Diptera |
| Suborder | Brachycera |
| Infraorder | Tabanomorpha |
| Superfamily | Tabanoidea |
| Family | Tabanidae |
| Subfamily | Tabaninae |
| Tribe | Tabanini |
| Genus | *Tabanus* Linneaus, 1758 |

The females of these flies are haematophagous and when present in large numbers can cause damage to man and livestock through blood loss, annoyance and disease transmission (Granett *et al.* 1957; Hollander and Wright 1980a; Perich *et al. 1986*). Female *T. mularis* flies

are telmophages and imbibe an average blood meal of 56.9 mg showing specificity for attacking cattle on the legs, underline, and neck (Hollander and Wright 1980a). They also had the highest engorgement rate of any species of horse fly in northcentral Oklahoma at 32% (Hollander and Wright 1980a). This means that fewer *T. mularis* flies were disloged by the cow before completion of a blood meal. This high engorgement rate coupled with the fact that these are anautogenous flies (requiring a blood meal prior to each oviposition) makes them potential mechanical transmitters of disease.

Krinsky (1976) reviewed the role of the family Tabanidae as mechanical transmitters of disease. Although *T. mularis* has never been incriminated in transmitting diseases, some of the infectious agents transmitted by the greenhead complex include *Anaplasma marginale* Theiler, *Bacillus anthracis* Sterne, Equine infectious anemia virus, Hog cholera virus, *Trypanosoma evansi* Steel, *Trypanosoma theileri* Laveran, and Western Equine encephalitis virus. Additionally, wounds resulting from horse flies feeding on infected animals are sources of infection from which transfer of disease agents to healthy animals may occur via other arthropods. Examples of this include the non-biting muscoid flies and members of the family chloropidae (*Hippelates* spp.) which associate with Tabanidae feeding on cattle (Roberts 1968).

## Cuticular Hydrocarbons

Cuticular lipids are important to the survival of insects for a number of reasons. They are a major barrier to water loss; they affect the absorption of agricultural chemicals; they prevent microorganisms from attaching to and penetrating the cuticle; they function as pheromones for aggregation and sex attraction and play roles in cast recognition cues (Blomquist *et al.* 1987; Bergman *et al.* 1990 ). Extensive reviews of insect cuticular lipids are given by Lockey (1988) and Blomquist and Dillwith (1985). Among the lipid constituents which comprise the cuticular lipid of insects are fatty acids, alcohols, esters, glycerides, sterols, aldehydes, ketones and hydrocarbons (Lockey 1988).

Hydrocarbons are synthesized in specialized epidermal cells called oenocytes which are associated with the peripheral fat body. These cells are modified for lipid metabolism and synthesize hydrocarbons from acetate. Once synthesized, the hydrocarbons are transported through the insect haemolymph by the carrier protein lipophorin. This carrier protein acts as a reusable shuttle, loading the hydrocarbons at the oenocyte surface and unloading them at the epidermis. Hydrocarbons are carried in the center of the molecule surrounded by protein and phospholipid and are thus transported through the aqueous haemolymph. Lipophorin does not deliver the hydrocarbons directly to the cuticle, this is accomplished through pore canals which extend from the epidermis to the inner epicuticle. The pore canals empty into epicuticular channels which perforate the outer epicuticle. It is through these epicuticular channels that the hydrocarbons are excreted (Lockey 1988).

Insects probably obtain some of their cuticular hydrocarbons, for example n-alkanes, directly from their diet (Blomquist and Jackson 1973), though biochemical studies using labelled precursors, confirm the view that insects synthesize most of their hydrocarbon constituents (Lockey 1980). Hydrocarbons are synthesized by the elongation-decarboxylation pathway from fatty acids (Major and Blomquist 1978). Cuticular hydrocarbons often occur as complex mixtures of straight-chain hydrocarbons (n-alkanes), unsaturated hydrocarbons (olefins) and methyl-branched hydrocarbons (methylalkanes) which are found in the wax or lipid layer of the epicuticle.

Hydrocarbons and other lipids are usually obtained by extracting whole non-homogenized bodies of insects with an organic solvent such as hexane. Extraction procedures aim at removing all cuticualr lipids, without removing internal body lipids (Lockey 1988). It is important to note that hydrocarbons are also found in the haemolymph and internal tissues and that the types of components found internally may differ from those on the surface (Blomquist *et al.* 1987). One of the problems involved with the extraction of cuticular lipids has been the lack of a standard solvent or time period of immersion. Another has been the lack of data confirming that only the surface lipids have been extracted.

## Research Efforts Related to Present Study

Since insects generally synthesize most of their hydrocarbons, hydrocarbon composition is expected to be a reflection of the genotype that should be useful in taxonomic grouping (Lockey 1980). In this type of analysis the hydrocarbon extract is injected onto the gas chromatograph and a profile is produced in which each type of hydrocarbon molecule appears as a peak. Multivariate statistics are applied to determine differences among samples of flies based on concentrations of the extracted components. Hydrocarbon analysis has been refined as a taxonomic tool to assist in identification of medically important Diptera such as black flies, sand flies and mosquitoes. It is also used as an investigative method to differentiate among strains within several species of Diptera. Hydrocarbon analysis is suited for exploratory studies when trying to determine how many distinct groups are represented in a set of samples (Phillips *et al.* 1988). Cuticular hydrocarbon analysis is a biochemical identification technique which is quick, inexpensive and is a nondestructive process that allows specimens to be retained as voucher specimens for future references. This differs from identification techniques based on separation of chromosomes, enzymes or DNA which require destruction of the specimen.

Philips *et al.* (1988) report that pinned, museum specimens can be used in this type of analysis, and that the presence of napthalene in storage boxes does not interfere. Furthermore, they report that in flies of different ages, there are differences only in total hydrocarbon content. A recently emerged fly may have the same hydrocarbons as at one-week after emergence, but in a reduced amount. This differs from the findings of Dillwith *et al.* (1981) who report dramatic changes in the production of hydrocarbon components of the house fly sex pheromones with the age of the insect. Toolson and Simbron (1989) report long-term maintenance under laboratory conditions leading to significant changes in epicuticular hydrocarbons of male and female fruit flies.

The use of cuticular hydrocarbon profiles of insects as taxonomic characters was first suggested by Jackson and Blomquist (1976) and Lockey (1976). Lockey (1978) was the first to demonstrate the utility of this type of analysis in species identification by demonstrating

consistent quantitative differences in the hydrocarbons between closely related species. Carlson and Service (1979) were the first to use hydrocarbons to distinguish between sibling species of Diptera. They distinguished the mosquito *Anopheles gambiae* Giles from *A. arabiensis* Patton, both of which are members of the *Anopheles gambiae* complex.

The *Anopheles maculipennis* Meigen complex has been analyzed by Phillips *et al.* (1990). Chen *et al.* (1990) used cuticular hydrocarbon profiles to separate different geographic strains of *Culex quinquefasciatus* Say. Kruger *et al.* (1991) and Kruger and Pappas (1993) used this technique to distinguish among 14 geographic variants of *Aedes albopictus* (Skuse). Carlson and Walsh (1981) and Phillips *et al.* (1985) used cuticular hydrocarbon compositions to differentiate two black flies of the *Simulium damnosum* Theobald complex, *S. sirbanum* Vajime and Dunbar and *S. sqamosum* Enderlein (Simuliidae). Ryan *et al.* (1986) and Philips *et al.* (1990) used hydrocarbon profiles to distinguish the Psychodidae *Psychodopygus carrerai carrerai* Barreto and *P. yucumensis* lePont, Caillard, Tibayrenc and Desjeux. Kamhawi *et al.* (1992) showed differences in cuticular hydrocarbons of *Phlebotomus argentipes* Annandale and Brunetti (Diptera: Phlebotominae) from field populations in northern India and Sri Lanka. Jallon and David (1987) analyzed the cuticular hydrocarbons of eight species of the *Drosophila melanogaster* Meigen subgroup and inferred ancestral and derived patterns, while Bartelt *et al.* (1986) used hydrocarbon profiles to construct a phylogeny of the *Drosophila virilis* Sturtevant species group.

Nelson *et al.* (1988) used hydrocarbons to separate the tsetse flies *Glossina fuscipes fuscipes* Newstead, *G. palpalis palpalis* Robineau-Desvoidy, *G.p. gambiensis* Vanderplank, *G. tachinoides* Westwood and *G. brevipalpis* Newstead. Carlson *et al.* (1993) used this technique to distinguish between 26 species and subspecies of tsetse flies (*Glossina* spp.) some of which cannot be identified by standard morphological techniques. Carlson and Yocom (1986) identified six species of Tephritid fruit flies based on this type of analysis. Lastly, Hoppe *et al.* (1990) used this analysis to show differences in the cuticular hydrocarbon composition of the horse flies *T. mularis* ,*Tabanus equalis* Hine, *T. abactor* Philip, *T. nigripes* Wiedemann, *T. venustus*

OstenSacken, *T. calens* L., *T. atratus* F., *and T. stygius* Say. This has been the only study to use cuticular hydrocarbon analysis on the Tabanidae to date. A more detailed analysis of cuticular hydrocarbon extracts of the three morphologically similar species *T. abdominalis* F., *T. sulcifrons* Macquart, and *T. limbatenevris* Macquart showed distinct hydrocarbon profiles for each species (Hoppe *et al.* 1990).

The type of statistical analysis which is applied to the hydrocarbon data is canonical discriminant analysis. This type of analysis has been used by Ryan *et al.* (1986) in separating female *Psychodopygus wellcomei* Fraiha, Shaw and Lainson, the vector of leishmaniasis in Brazil, and *P. complexus* Mangabeira. Kruger *et al.* (1991) found geographic variation among seven North American populations of *Aedes albopictus*. This study was followed up by a study in 1993, where Kruger and Pappas found geographic variation among 14 populations of *Aedes albopictus* using canonical discriminant analysis. Golden *et al.* (1992) used canonical discriminant analysis to separate the sibling species *Diabrotica longicornis* Smith and Lawrence and *D. barberi* Smith and Lawrence (Coleoptera: Chrysomelidae) from sympatric and allopatric populations.

## CHAPTER III

## Preliminary Studies on Cuticular Hydrocarbons of *T. mularis* and *T. quinquevittatus*

### Introduction

Numerous authors have used cuticular hydrocarbon analysis to differentiate between closely related groups of insects. Phillips *et al.* (1988) have reviewed the use of this type of analysis in the medically important Diptera. There is a need to determine if specimens of the horse flies identified as *T. mularis* in Oklahoma constitute a single group or belong to several strains within the species. Therefore, a preliminary study was designed to determine whether the analysis of cuticular hydrocarbons could be applied to differentiate between *T. mularis* and a closely related species *T. quinquevittatus.* Due to unexplained variability in the composition of the cuticular hydrocarbons of the *T. mularis* species, a second experiment was designed to test the effect of length of time stored and handling conditions on the composition of extractable hydrocarbons of *T. mularis.*

### Methods and Materials

Field specimens of *T. mularis* (Fig. 1) were collected at the Oklahoma State University Entomology Pasture Two Cross-Timbers Experimental Range (CTER) in Township 18N, located approximately 11 km southwest of Stillwater in Payne County, Oklahoma. This cross-timbers vegetation is sometimes referred to as oak savannah or upland forest. The 32 ha CTER pasture two is composed of 60% upland forest, comprised mainly of blackjack oak (*Quercus marilandica* Muenchh) and post oak (*Quercus stellata* Wangenh) and 40% tallgrass prairie of little bluestem (*Andropogon scoparius* Michx), Indian grass (*Sorghastrum nutans* (L.) Nash) and Rosette panicgrass (*Panicum oligosanthes* (Nash) Fernald) (Ewing *et al.* 1984). The four characteristic soil mapping units occurring in this pasture were 11-Stephenville-Darnell complex, 1 to 8% slopes; 26-Grainola-Lucien complex, 3 to 5% slopes; 32-Harrah-Pulaski complex, 0 to 8% slopes and 49-Renfrow and Grainola soils, 3 to 8% slopes; eroded (Ewing *et al.*1984).

Field specimens of *T. mularis* were also collected from lake Carl Blackwell, Payne County, OK. Flies were collected in modified Stoneville Malaise traps (Fig. 2) (Wright *et al.* 1986). This type of trap was reported by Roberts (1976) to be the most effective for collecting

Fig 1. Specimen of a female *T. mularis* .

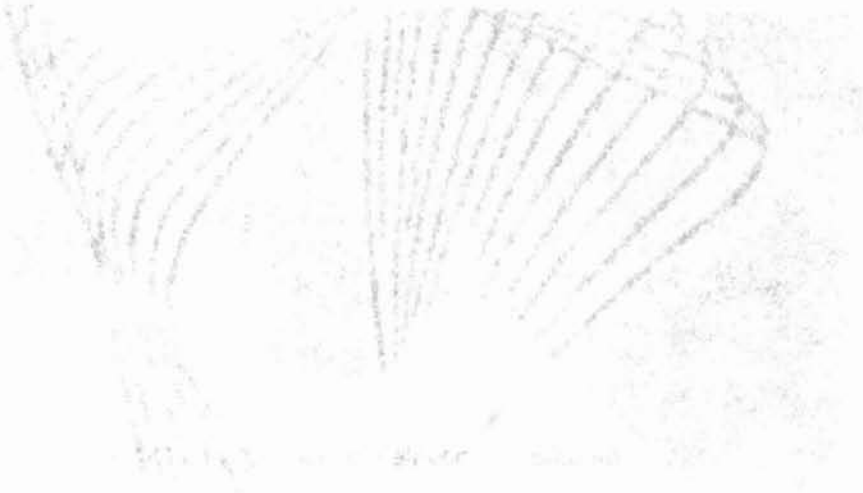


Fig 2. Modified Stoneville Malaise trap with $CO_2$ cylinder.

Tabanidae. Traps were baited with compressed $CO_2$ released from cylinders through two stage regulators at the rate of 200 cc/minute. Leprince (1986) found that carbon dioxide increased by 9-fold the number of trapped *T. quinquevittatus* over traps without $CO_2$, and Knox *et al.* (1972) found similar results with other Tabanidae. Inverted funnel containers were placed at the top of the Malaise trap each with a 2.5 $cm^2$ piece of dichlorvos-impregnated resin to quickly kill the trapped flies.

*Tabanus mularis* and *T. quinquevittatus* flies were identified using the taxonomic key in the Diptera, or true flies of Illinois (Pechuman et al. 1983). *Tabanus mularis* is differentiated from *T. quinquevittatus* by coloration of hairs on the pleuron and labial palpi. The hairs of *T. mularis* have a white appearance while those of *T. quinquevittatus* appear creamy yellow.

Experiment 1

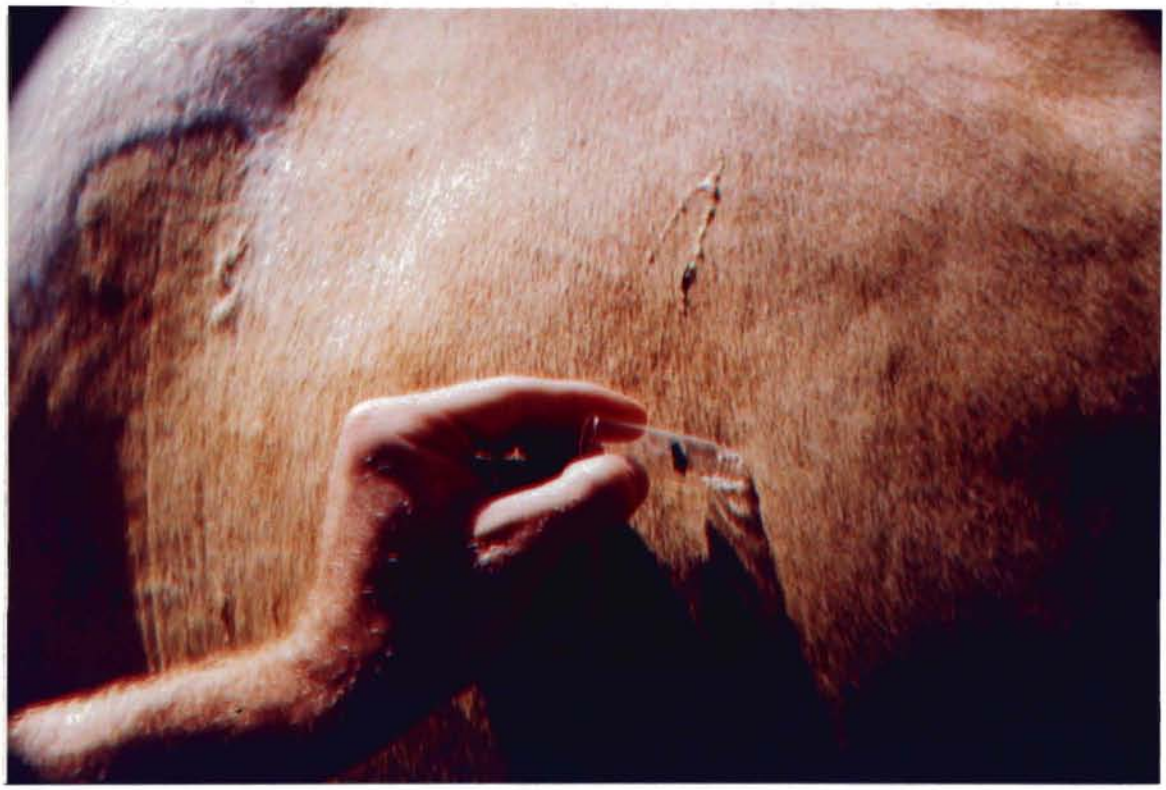
A study was designed to determine if cuticular hydrocarbon extracts would differentiate between *T. mularis* and *T. quinquevittatus*. Female *T. mularis* flies from the field (TmF) were collected from CTER pasture 2 directly from tethered cattle by placing a clean 7ml scintillation vial over each fly when feeding (Fig. 3). The flies were then chilled on ice and immediately returned to the lab for extraction of the hydrocarbons. Pinned *T. quinquevittatus* flies (TqM) were obtained from the K.C. Emerson museum at Oklahoma State University and were identified by the late L.L. Pechuman. Pinned *T. mularis* flies (TmM) were obtained from the K.C. Emerson museum at Oklahoma State University and were also verified by L.L. Pechuman.

Cuticular hydrocarbons of pinned *T. quinquevittatus* from the K.C. Emerson museum were compared with cuticular hydrocarbons of pinned *T. mularis* from the K.C. Emerson museum and cuticular hydrocarbons of *T. mularis* which were freshly collected in scintillation vials in the field.

Experiment 2

A temporal study was conducted by comparing cuticular hydrocarbons of museum specimens of *T. mularis* collected from lake Carl Blackwell in 1977, 1978, and 1979 with those fresh specimens of T. mularis collected from lake Carl Blackwell in 1995 collected in malaise

Fig 3. Collection of female *T. mularis* with 7ml borosilicate glass scintillation vial.



traps or fresh specimens which were pinned for four months. This experiment was designed to determine the effect of the length of time of storage and handling conditions on the analysis of cuticular hydrocarbons of *T. mularis*.

## Cuticular Lipid Extraction

Methods followed those used by Hoppe *et al.* (1990) with some modifications. All hexane used was redistilled in glass, and all glassware was rinsed with hexane before use. Cuticular lipids were obtained from single horse flies by placing the flies into a clean 7 ml glass scintillation vial along with an internal standard of 3.125 µg n-hexacosane (chosen because this alkane was absent from the cuticular hydrocarbons) and exposing the flies to two 30 second extractions of 3.5 ml of hexane. The vials were gently swirled during the extractions. The two 3.5 ml hexane fractions were then combined in one 7 ml scintillation vial, and the vial was placed in a 60$^0$C sand bath and dried under a stream of lamp grade nitrogen. Lipids were redissolved in hexane prior to silica column chromatography.

## Hydrocarbon Isolation

Hydrocarbons were isolated from the lipid extracts by dissolving the sample in 1 ml hexane and transferring to a silica gel column, (7 cm x 0.5 cm i.d.) 100-200 mesh (70-140 µ), average pore diameter 25 Å (Sigma Chemical Co., St. Louis , MO), which had been pre-washed with 6 ml hexane. Hydrocarbons were eluted from the column with 7 ml hexane, dried under nitrogen and stored at room temperature until analyzed (Blailock *et al.* 1976; Nelson *et al.* 1981).

When examining hydrocarbons of *T. mularis* or any other horse flies the researcher must be aware of the possibility that the insects may have been immersed in an organic solvent. For many years it has been commonplace for systematists studying the Tabanidae to degrease specimens (David Carlson & Richard Roberts, Florida State Univ., Gainesville Fla.; personal comm.). This is especially true of museum specimens. Degreasing is accomplished by immersing the flies in some organic solvent with the intention of cleaning the specimens to examine subtle differences in coloration of areas such as the eyes, and hairs of the palpi and

pleural regions. This process apparently aids in taxonomic classification, but unfortunately renders the specimens useless for hydrocarbon analysis.

## GC Analysis

Hydrocarbon analysis was performed using a Hewlett Packard 5890 II gas chromatograph. Samples were redissolved in 50 µl hexane, and 1µl was injected onto a 100% methyl silicone capillary column (DB-1, 15m, .25 mm i.d., 0.1 µm film thickness, J&W Scientific), using a splitless injector at 250°C and a detector at 350°C. Thus, the equivalent of 2% of the cuticular hydrocarbons was injected. The temperature program consisted of an initial temperature of 200°C, increasing by a ramp of 5°C /minute to a temperature of 325°C. The signal was collected on and analyzed by Maxima Software® (Dynamic Solution, Milford, MA).

## Statistical Analysis

The analytical technique applied to the cuticular hydrocarbon patterns was one which compared areas under some selected peaks of the chromatograms. Each chromatogram was examined individually , and each major peak was characterized by its retention time and total area. Thus, each GC peak selected, was used as a "character" for phenetic analysis. Peaks were provisionally identified from their retention times and numbered so that the same peak in different individuals could be determined. The area under each peak, found by integration, was proportional to its concentration in the injected sample and was compared to the area for the internal standard. The internal standard was of known concentration and, when injected with each sample, gave the constant of proportionality; it also standardized runs by eliminating the worry of differences in injection volume and machine response (Phillips et al. 1988).

The raw data for analysis consisted of the area under the selected major peaks divided by the total area of the hydrocarbons represented in a chromatogram. Twenty-eight hydrocarbon peaks were selected for *T. mularis* of which, twelve were considered as major peaks which occurred in each chromatogram. Eleven major peaks were considered for *T. quinquevittatus*. Peak areas selected were scaled or normalized (summed to 100%), thereby considering the area under the 28 chosen peaks to represent all of the hydrocarbons. Minor peaks were ignored. A

canonical discriminant analysis was used to differentiate between groups of flies. This analysis is a dimension-reduction technique related to principal component analysis and canonical correlation. Given several quantitative variables (hydrocarbon peaks) this procedure derives *canonical variables* (linear combinations of the quantitative variables) that summarize between-class variation. A multivariate analysis of variance was performed using Proc GLM (SAS Institute Inc. 1989). This procedure performs multivariate analysis of variance (MANOVA) and computes squared distances between class means based on the pooled within-class covariance matrix (Mahalanobis distances) (SAS Institute Inc. 1989). Four statistical tests including Wilks Lambda, Pillai's trace, Roys greatest root and Hotelling-Lawley trace confirm significant differences between groups.

Given two or more groups of observations with measurements on several quantitative variables, canonical discriminant analysis derives a linear combination of the variables that has the highest possible multiple correlation with the groups. This maximal multiple correlation is called the *first canonical correlation*. The coefficients of the linear combination are the *canonical coefficients* or *canonical weights*. The variable defined by the linear combination is the *first canonical variable* or *canonical component*. The second canonical correlation is obtained by finding the linear combination uncorrelated with the first canonical variable that has the highest possible multiple correlation with the groups. This process of extracting canonical variables is repeated until the number of canonical variables equals the number of classes minus one. (SAS Institute Inc. 1989).

Canonical discriminant analysis is also equivalent to performing the following steps:

1. Transform the variables so that the pooled within-class covariance matrix is an identity matrix.
2. Compute class means on the transformed variables.
3. Do a principal component analysis on the means, weighting each mean by the number of observations in the class. The eigenvalues are equal to the ratio of between-class variation in the direction of each principal component.
4. Back-transform the principal components into the space of the original variables,

obtaining the canonical variables. (SAS Institute Inc. 1989).

## RESULTS AND DISCUSSION

When the external hydrocarbons of the three groups of flies were compared by general linear models procedure (SAS Institute Inc. 1989), there were significant differences among all three groups in the MANOVA Wilkes Lambda test (F=30.9655; DF=26; Pr>F 0.0001). The cuticular hydrocarbon profiles of *T. quinquevittatus* were obviously different from those of either group of *T. mularis*. However, the hydrocarbon profiles of the two groups of *T. mularis* were also significantly different from each other. Figure 4 depicts a plot of the first two canonical discriminant variables from the analysis of the cuticular hydrocarbons of *T. mularis* and *T. quinquevittatus*. Canonical axis 1 separates *T. mularis* from *T. quinquevittatus*. Canonical axis 2 separates the two groups of *T. mularis*. When the discriminant analysis was executed, the SAS program correctly identified 97% of the flies. Two *T. mularis* from the museum were misidentified as coming from the field. The difference of the cuticular hydrocarbon profiles within the *T. mularis* specimens appeared to be attributed to the conditions the flies were exposed to prior to hexane extraction.

Based on the results of the first study, it was necessary to determine if the differences in the cuticular hydrocarbon profiles of the *T. mularis* specimens were real or artifacts of storage conditions. To confirm the role that the storage conditions were playing, if any, in the variability of the *T. mularis* group, a second experiment was conducted. Figure 5 shows a two-dimensional canonical discriminant analysis of *T. mularis* flies from the late 1970's and those collected from 1995. The flies from 1995 were collected from the same location as those from the late 1970's. Canonical axis 1 separates the flies from 1977, 1978, and 1979 which were taken from the museum from those which were collected in the field in 1995. Canonical axis 2 separates the 1995 flies into two groups based on storage conditions prior to extraction of the hydrocarbons with hexane. When the external hydrocarbons of the three groups of flies were compared by general linear models procedure (SAS Institute Inc. 1989), there were significant differences

Fig. 4 Canonical discriminant analysis of cuticular hydrocarbons from museum specimens of *T. mularis* (TmM), freshly extracted specimens of *T. mularis* (TmF), and museum specimens of *T. quinquevittatus* (TqM).

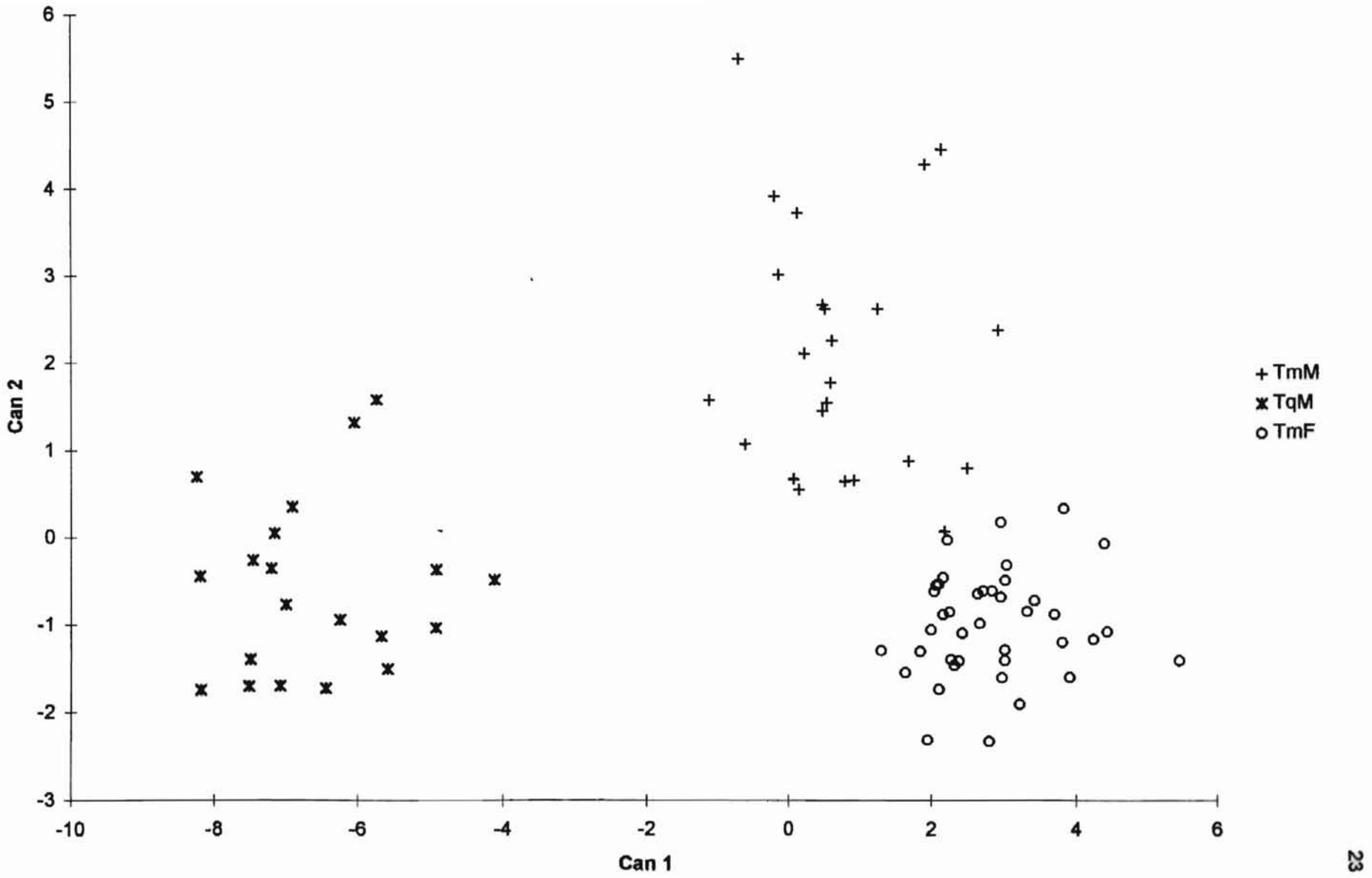
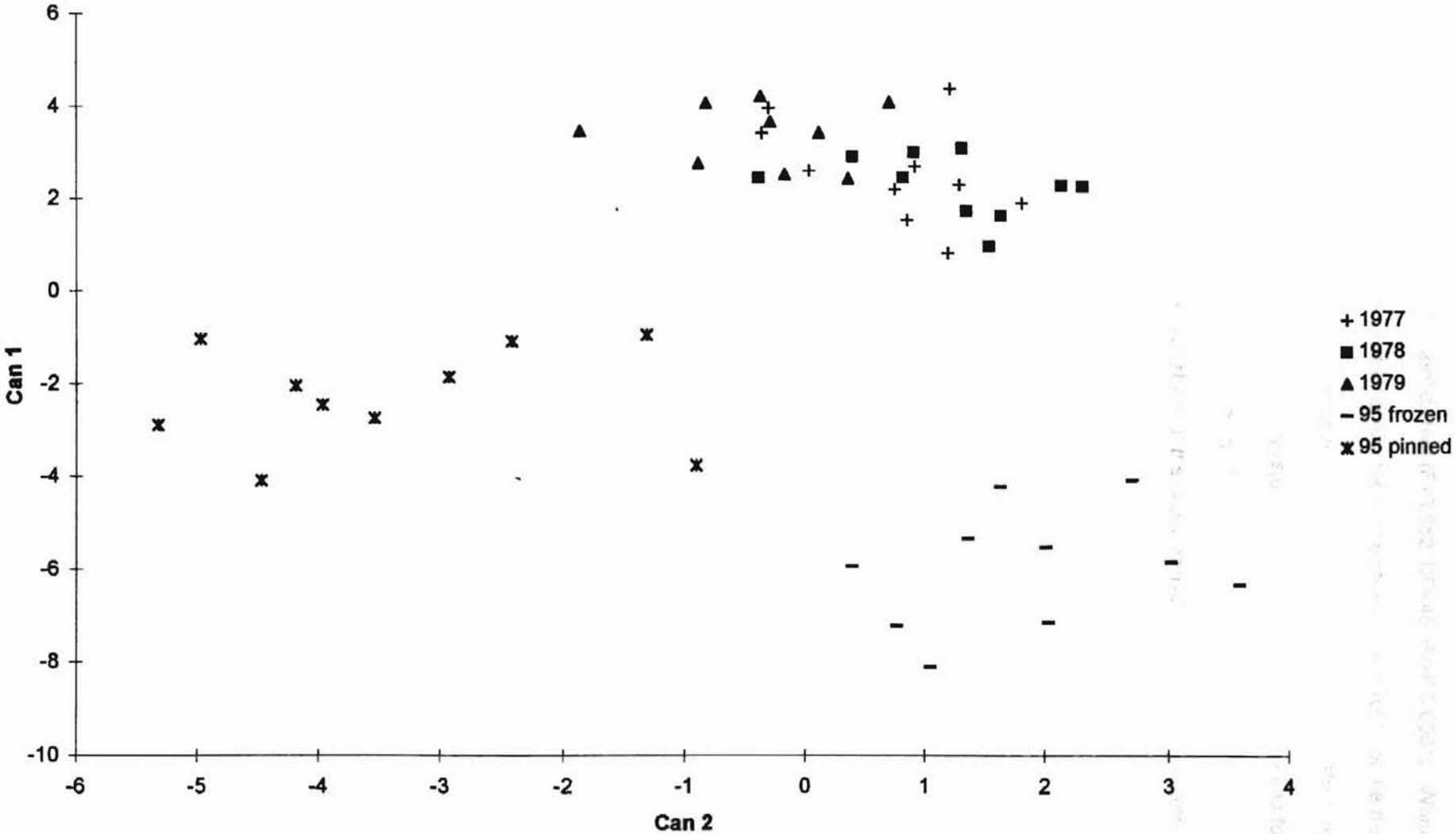

6
5
4
3
2
1
0
-1
-2
-3
Can 2
-10
-8
-6
-4
-2
0
2
4
6
Can 1
+ TmM
* TqM
o TmF

Fig. 5 Canonical discriminant analysis of cuticular hydrocarbons from *T. mularis* from Lake Carl Blackwell, Payne County OK. Specimens from 1977, 1978, 1979 were stored in Cornell specimen drawers with naphthalene. Specimens collected in 1995 were pinned for 4 months or frozen for 3 months before extraction.


6
4
2
0
-2
-4
-6
-8
-10
Can 1
-6
-5
-4
-3
-2
-1
0
1
2
3
4
Can 2
+ 1977
■ 1978
▲ 1979
– 95 frozen
✱ 95 pinned

between groups in the MANOVA Wilkes Lambda test (F=6.952; DF=48; Pr>F 0.0001). When the discriminant analysis was executed, the SAS program correctly identified 100% of the flies.

The results of these two experiments strongly suggested that something was affecting the amount of extractable hydrocarbons of *T. mularis* stored under different conditions and for differing time periods. Once this was determined, the focus of this study shifted to try to further elucidate this point and to examine the variability of the cuticular hydrocarbons of T. mularis, if any, with respect to seasonal distribution and geographic location.

## CHAPTER IV

## The Effects of Storage Conditions on the Use of Cuticular Hydrocarbon Analysis for Identification of *T. mularis*

### Introduction

The preliminary studies indicated that there were significant differences in the cuticular hydrocarbon profiles of *T. mularis* females that were stored under different conditions prior to hexane extraction. A study was designed to gather baseline information about the cuticular hydrocarbons of freshly collected T. *mularis.* This included a partial characterization of the hydrocarbons, as well as a determination if any internal hydrocarbons were being extracted from the flies. The baseline information obtained from the fresh specimens was used in comparison with flies stored under different conditions.

### Methods and Materials

The first step in the analysis was to gather baseline information about the cuticular hydrocarbons of freshly collected *T. mularis* to compare with cuticular hydrocarbon profiles of *T. mularis* stored under different conditions. This included a partial characterization of the cuticular hydrocarbons, and a determination of the amounts of extractable cuticular hydrocarbons from flies stored under different conditions prior to hexane extraction. Once this was accomplished, it was determined if any internal hydrocarbons were being extracted from the *T. mularis* specimens.

On 14 June 1995, 100 *T. mularis* were collected from CTER pasture 2 ( 80 Specimens freshly collected from tethered cattle, and 20 specimens from malaise traps). These specimens were separated into five groups, each containing 20 flies exposed to different storage conditions. The storage conditions were as follows:

1. 20 fresh specimens ( which were chilled to immobilize prior to extracting hydrocarbons)

2. 20 lyophilized specimens ( freeze dried, then immediately extracted)
3. 20 pinned specimens (pinned 4 months)
4. 20 frozen specimens (frozen 3 months)
5. 20 specimens from a malaise trap

Each group of 20 flies was subdivided into 2 groups of 10 specimens for analysis. The first group was homogenized and extracted using the method of Bligh and Dyer (1959) to give a total lipid fraction. Single horse flies were homogenized in 3.8 ml of a mixture of redistilled chloroform: methanol: water, 1:2:0.8 (volume/volume) along with 3.125 µg n-hexacosane as an internal standard. The mixture was transferred to a test tube and combined with 1ml redistilled chloroform ($CHCl_3$)and 1ml $H_20$. The mixture was then vortexed for 30 seconds, followed by a 10 minute centrifugation ( 850 x g). The bottom $CHCl_3$ layer was removed and saved in a 7 ml scintillation vial, and 1ml redistilled $CHCl_3$ was added to the test tube. The mixture was again vortexed for 30 seconds, followed by a 10 minute centrifugation (850 x g). The chloroform fractions were combined in the 7 ml scintillation vial and dried under nitrogen giving the internal lipid fraction.

The cuticular hydrocarbons were extracted from the second group of specimens with hexane as described in chapter two. In order to avoid extraction of any internal hydrocarbons, two short extraction periods (30 sec.) were used. This group of specimens was then homogenized and the internal lipids were obtained using the Bligh and Dyer method of extraction. The statistical analysis used for this study was the same as described in the previous chapter.

## HPTLC

High performance thin layer chromatography was used to confirm separation and purification of eluted hydrocarbon fractions of *T. mularis*. Samples containing isolated hydrocarbon and total lipid fractions from single flies were run with standards containing monoglyceride, cholesterol, diglyceride, free fatty acid, triglyceride, wax ester, and n-alkanes. Merck 5626 HPTLC silica gel plates 60 (without fluorescent indicator, 10 cm x 20 cm, layer thickness 0.25 mm) were used. Plates were first developed in 100% redistilled hexane to the top

of the plate. The samples were then loaded to the plate. The plate was developed in a solvent system containing hexane, ether, acetic acid, 90:10:1 (volume/volume), to approximately 1 cm from the top of the plate. Lipids were visualized by treating plates with a mixture of 3% cupric acetate in 15% phosphoric acid and heating on a hot plate until spots appeared.

## Separation of Hydrocarbon Constituents

The total hydrocarbon fraction containing saturated, unsaturated and methyl-branched hydrocarbons was dissolved in 1 ml hexane and loaded to a silica gel column (Sigma: 7cm x 0.5cm i.d.) impregnated with silver-nitrate ($AgNO_3$) 20% (w/w) which had been pre-washed with 6 ml hexane. The saturated and methyl-branched fractions were obtained by rinsing the column with 7 ml of hexane. Unsaturated hydrocarbons (olefins = alkenes, alkadienes, & alkatrienes) were then eluted with 7 ml hexane: diethyl ether (1:1, v:v). The methyl-branched hydrocarbons were separated from the straight chain hydrocarbons (n-alkanes /paraffins) by dissolving this fraction in 2,2,4-Trimethylpentane (iso-octane) and adding a molecular sieve (5 Å) (O'Conner *et al.* 1962; Nelson *et al.* 1981). The molecular sieve was activated by heating to $300^0$C for 24 hours, and enough was added to cover 1ml of hexane. By doing this, the straight chain hydrocarbons are incorporated into the sieve, leaving the methyl-branched hydrocarbons behind.

## GC Analysis

Hydrocarbon analysis was performed using a Hewlett Packard 5890 II gas chromatograph. Samples were redissolved in 50 µl hexane, and 1µl was injected onto a 100% methyl silicone capillary column (DB-1, 15m, .25 mm i.d., 0.1 µm film thickness, J&W Scientific), using a splitless injector at 250°C and a detector at 350°C. Thus, the equivalent of 2% of the cuticular hydrocarbons was injected. The temperature program consisted of an initial temperature of 200°C, increasing by a ramp of 5°C /minute to a temperature of 325°C. The signal was collected on and analyzed by Maxima Software® (Dynamic Solution, Milford, MA).

## Equivalent Chain Lengths (ECL's)

The equivalent chain lengths (ECL) (Miwa 1963) were determined by injecting a mixture of known hydrocarbon standards (even chained n-alkanes C20-C40 1:1.....:1 (v/v) ) onto the GC and plotting their carbon chain lengths vs. retention times. ECL's of the flies hydrocarbons were then determined relative to these n- alkanes by linear interpolation.

## Results and Discussion

### HPTLC of *Tabanus mularis* Cuticular Lipid Extract

The HPTLC method allowed accurate separation of *T. mularis* cuticular lipids (fig 6). This method verified separation of the hydrocarbon constituents and also verified that hydrocarbon was the major lipid class present in freshly collected female *T. mularis*. Lane three shows the total lipid fraction of *T. mularis*, while lane four shows only the isolated hydrocarbons following application to the silica gel column.

### *Tabanus mularis* Hydrocarbons

Figure 7 shows a typical gas chromatogram for a freshly-collected female of *T. mularis* from which cuticular lipids were extracted with hexane while the fly was still living. The 28 peaks representing the cuticular hydrocarbons were present in all traces that were examined. Peaks marked with an asterisk were chosen as major peaks for the canonical discriminant analysis. Table 1 shows the corresponding equivalent chain lengths (ECL'S) for each of the numbered hydrocarbons shown in figure 7.

Blomquist *et al.* (1985) reported that previous research on Diptera has shown that the cuticular hydrocarbon components contain significant amounts of unsaturated components. Figures 8 and 9 show that this was not the case for *T. mularis*. When compared to the internal standard it was clear that the majority of cuticular hydrocarbons of *T. mularis* were saturated. This was consistent with the findings of Hoppe *et al.* (1990) for other species of horse flies.

Fig. 6 High performance thin layer chromatography plate showing the separation of *Tabanus mularis* cuticular lipids from freshly collected specimens.

Lane 1. Standard containing cholesterol (C), free fatty acid (FFA), monoglyceride (MG), diglyceride (DG), triglyceride (TG), sterol ester (SE), wax ester (WE), and hydrocarbon (HC).

Lane 2. Hydrocarbon (HC) standard.

Lane 3. *Tabanus mularis* total lipid extract.

Lane 4. Tabanus mularis isolated hydrocarbons.

Lane 5. Wax ester (WE) standard.

Lane 6. Standard containing (C), (FFA), (MG), (DG), (TG), (SE), (WE).

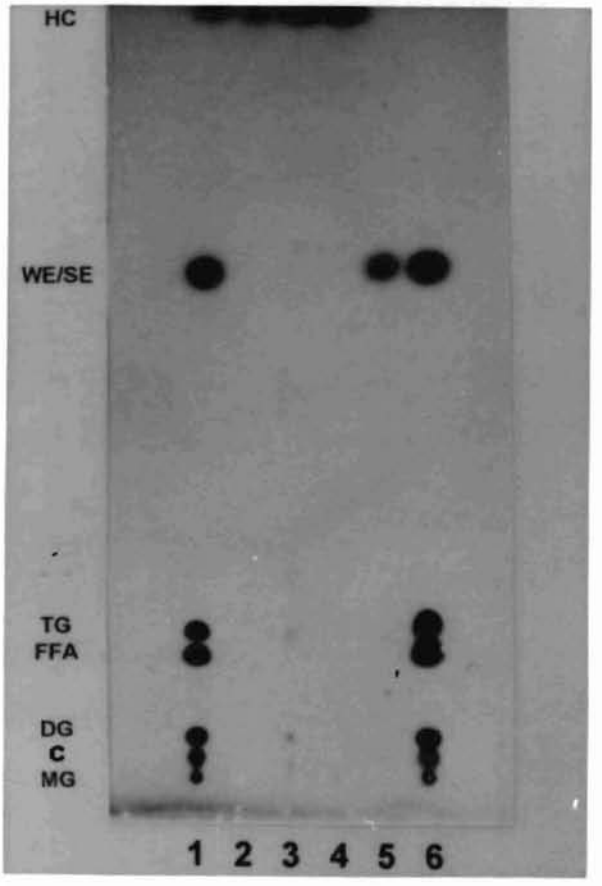
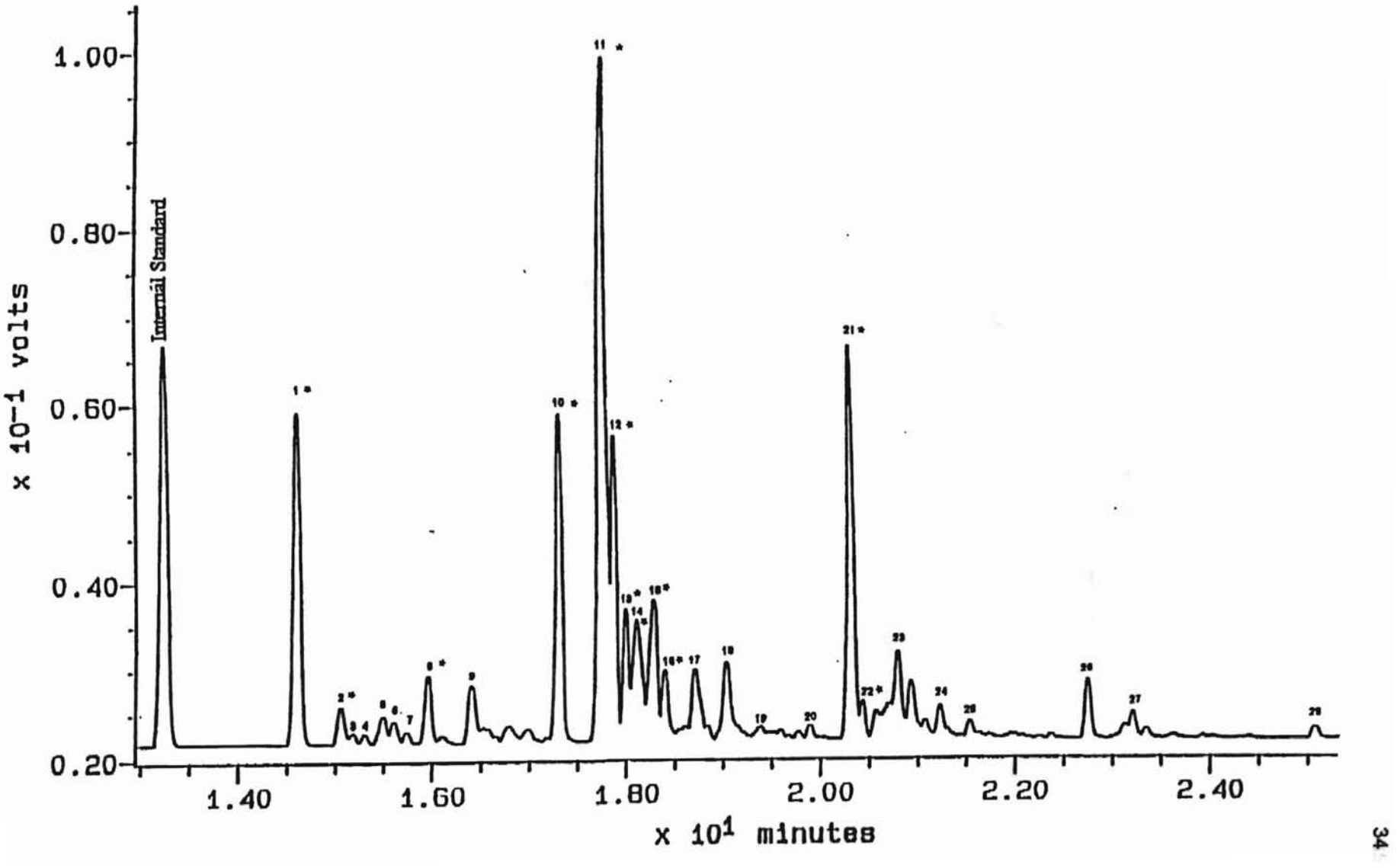

HC
WE/SE
TG
FFA
DG
C
MG
1 2 3 4 5 6

Fig. 7 Typical chromatogram of freshly extracted female *T. mularis* showing peaks chosen to represent total hydrocarbon fraction.


1.00
0.80
0.60
0.40
0.20
x 10⁻¹ volts
Internal Standard
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

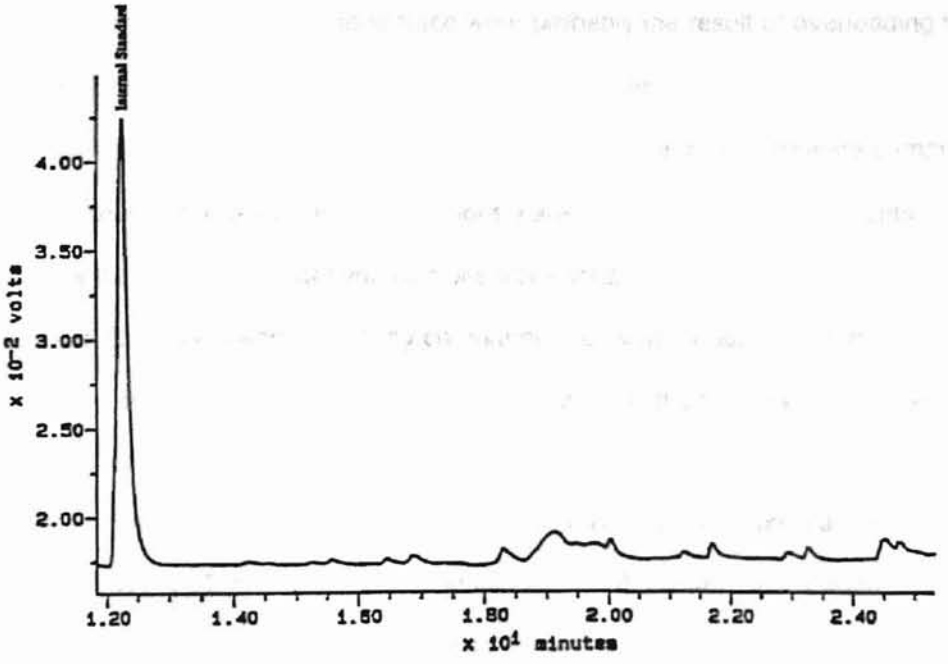
| Peak | Equivalent Chain Length |
|---|---|
| Internal Standard | 26 |
| Peak 1 | 27.29 |
| Peak 2 | 27.42 |
| Peak 3 | 27.49 |
| Peak 4 | 27.59 |
| Peak 5 | 27.72 |
| Peak 6 | 27.83 |
| Peak 7 | 28 |
| Peak 8 | 28.37 |
| Peak 9 | 29.08 |
| Peak 10 | 29.45 |
| Peak 11 | 29.56 |
| Peak 12 | 29.61 |
| Peak 13 | 29.7 |
| Peak 14 | 29.82 |
| Peak 15 | 29.96 |
| Peak 16 | 30.22 |
| Peak 17 | 30.45 |
| Peak 18 | 30.75 |
| Peak 19 | 31.15 |
| Peak 20 | 31.48 |
| Peak 21 | 31.64 |
| Peak 22 | 31.92 |
| Peak 23 | 32.49 |
| Peak 24 | 32.8 |
| Peak 25 | 33.48 |
| Peak 26 | 33.8 |
| Peak 27 | 34.53 |
| Peak 28 | 39.95 |

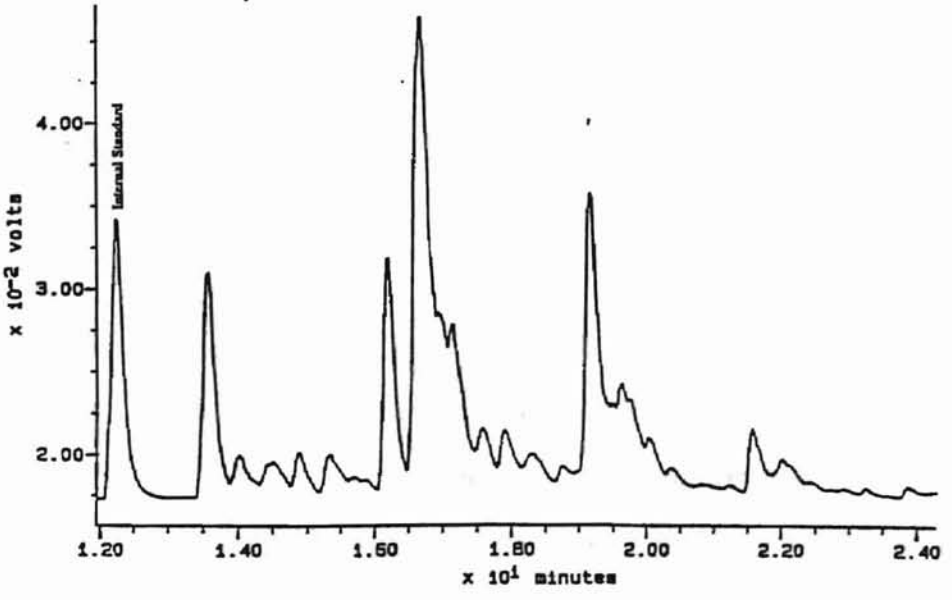
TABLE I

EQUIVALENT CHAIN LENGTHS OF HYDROCARBONS COMPRISING A TYPICAL CHROMATOGRAM OF A FRESHLY EXTRACTED FEMALE *T. MULARIS*

Fig. 8 Chromatogram of unsaturated hydrocarbons of *T. mularis*

Fig. 9 Chromatogram of saturated hydrocarbons of *T. mularis*


Internal Standard
4.00
3.50
3.00
2.50
2.00
x 10⁻² volts
1.20
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes


Internal Standard
4.00
3.00
2.00
x 10⁻² volts
1.20
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

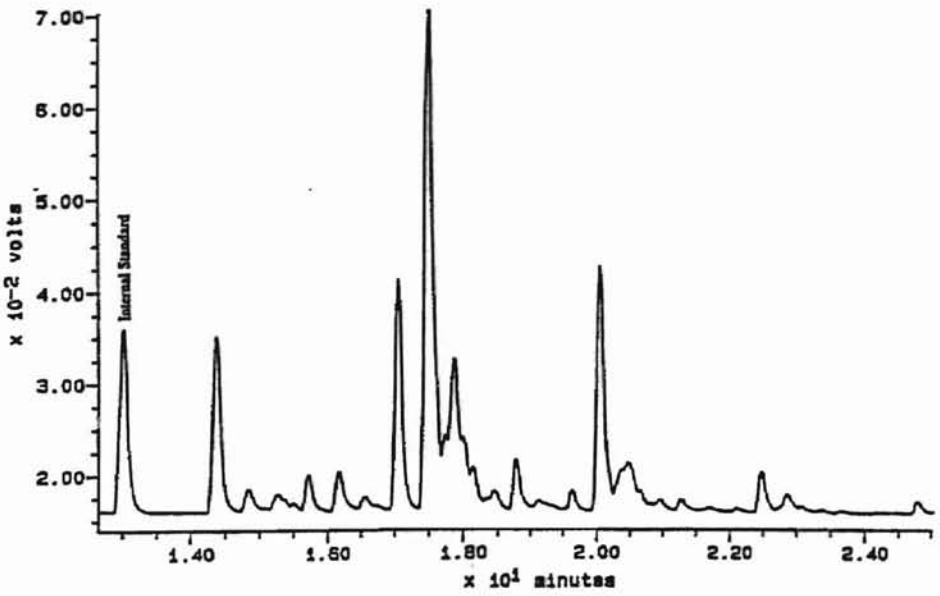
Small peaks occurring in the unsaturated trace were probably the result of overloading the silver-nitrate impregnated silica column with the hydrocarbon sample.

The cuticular hydrocarbons and internal hydrocarbons of *T. mularis* were comprised of the same components, however, the hydrocarbons were present in differing amounts. Figures 10 and 11 show that more cuticular hydrocarbons were obtained than internal hydrocarbons in freshly extracted flies. This was evident by comparing the hydrocarbons to the internal standard peaks. When profiles of cuticular hydrocarbons and internal hydrocarbons from the same 10 flies were compared by general linear models procedure (SAS Institute Inc. 1989) there were significant differences between the them as shown by the MANOVA Wilks' Lambda test ( F= 7.8241; DF=11; Pr >F 0.0036). In the discriminant analysis there were no misidentifications of the hydrocarbons between the two groups. Figure 12 illustrates this point by showing the distribution of the hydrocarbons in freshly extracted specimens. The bars represent means for three groups of flies (n=10 in each case). Originally, it was assumed that in freshly extracted *T. mularis* internal hydrocarbons were accumulated in the cuticular fraction due to permeability of the cuticle to the solvent hexane. However, further research clearly demonstrated that this was not the case. More will be discussed on this topic.

When storing female *T. mularis* under different conditions there were significant differences in the amount of cuticular hydrocarbons that could be obtained from hexane extractions. Figure 13 shows the results of five different processing methods prior to hexane extraction, on the amount of attainable cuticular hydrocarbons from female *T. mularis*. The bars represent means for the five groups of specimens (n=10 in each case). When specimens were extracted fresh or frozen for 3 months before extraction, the yield was over two times that of pinned and freeze dried specimens (Figures 14 and 15). The internal standard peak in each chromatogram represented 3.125 µg of the hydrocarbon n-hexacosane. When compared to the internal standard, there was much more cuticular hydrocarbon extracted from fresh specimens than pinned specimens. Figures 16 and 17 show that similar results were obtained when examining frozen or freeze-dried specimens. A typical chromatogram of a frozen specimen showed yields of approximately the same amount of cuticular hydrocarbons as a chromatogram

Fig. 10 Typical chromatogram of cuticular hydrocarbons of a freshly extracted female *T. mularis.*

Fig. 11 Typical chromatogram of internal hydrocarbons of a freshly extracted female *T. mularis.*


7.00
6.00
5.00
4.00
3.00
2.00
x 10⁻² volts
Internal Standard
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

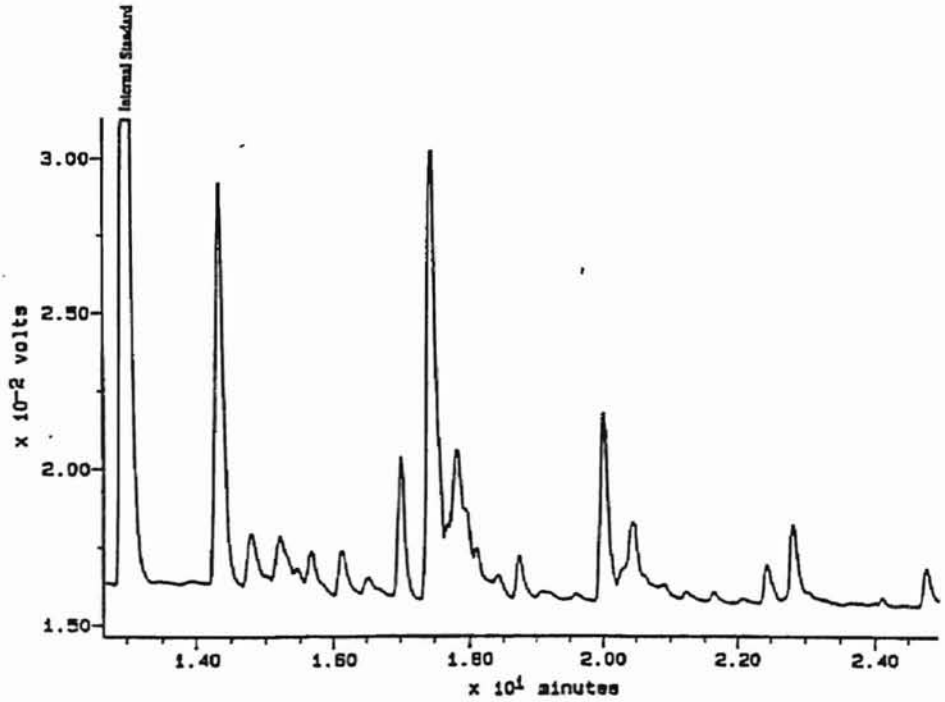

3.00
2.50
2.00
1.50
x 10⁻² volts
Internal Standard
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

Fig. 12 Distribution of the hydrocarbons of a freshly extracted *T. mularis*.

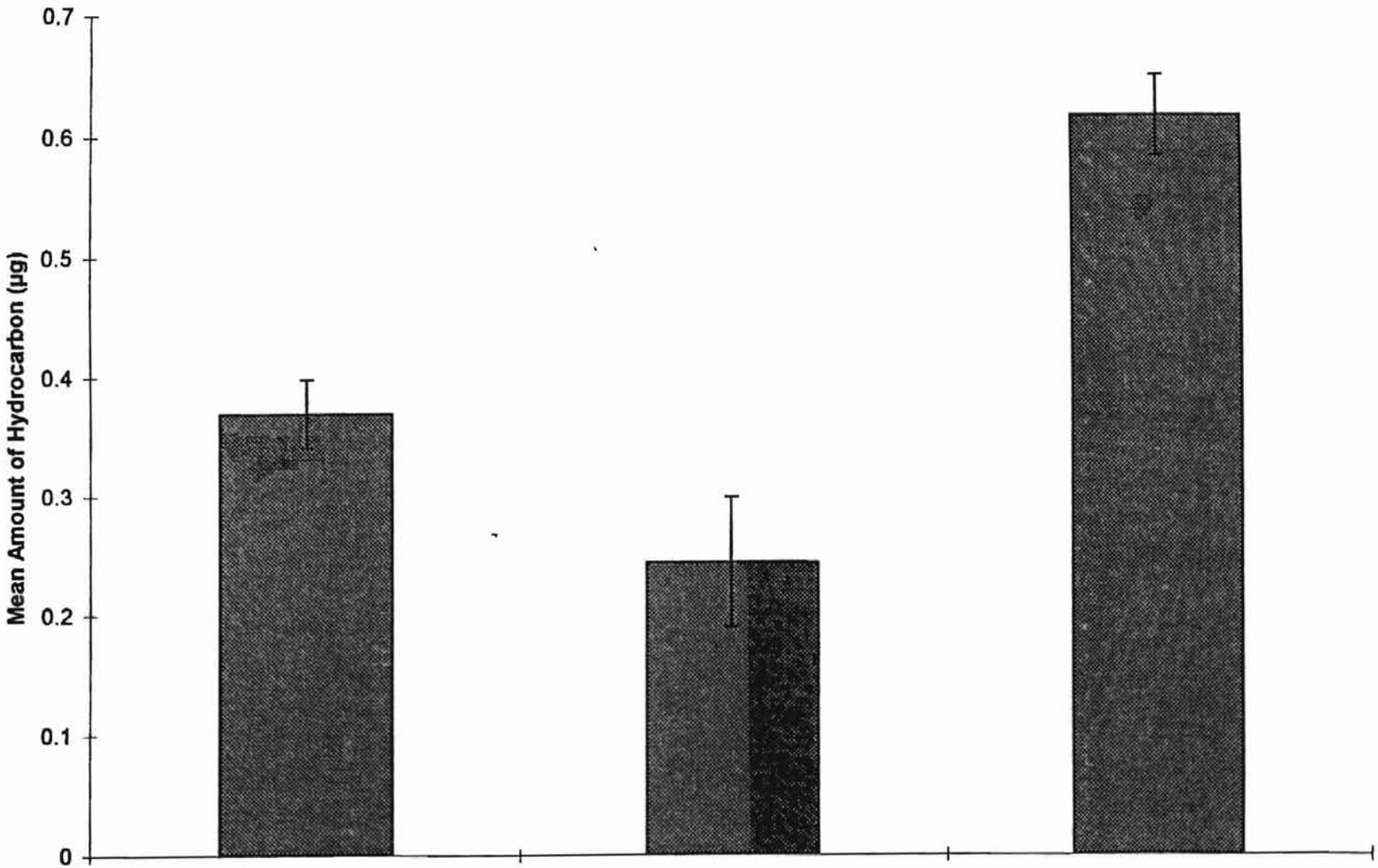

Mean Amount of Hydrocarbon (µg)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cuticular
Internal
Total

Fig. 13 Amounts of cuticular hydrocarbons extracted from *T. mularis* stored under different conditions.

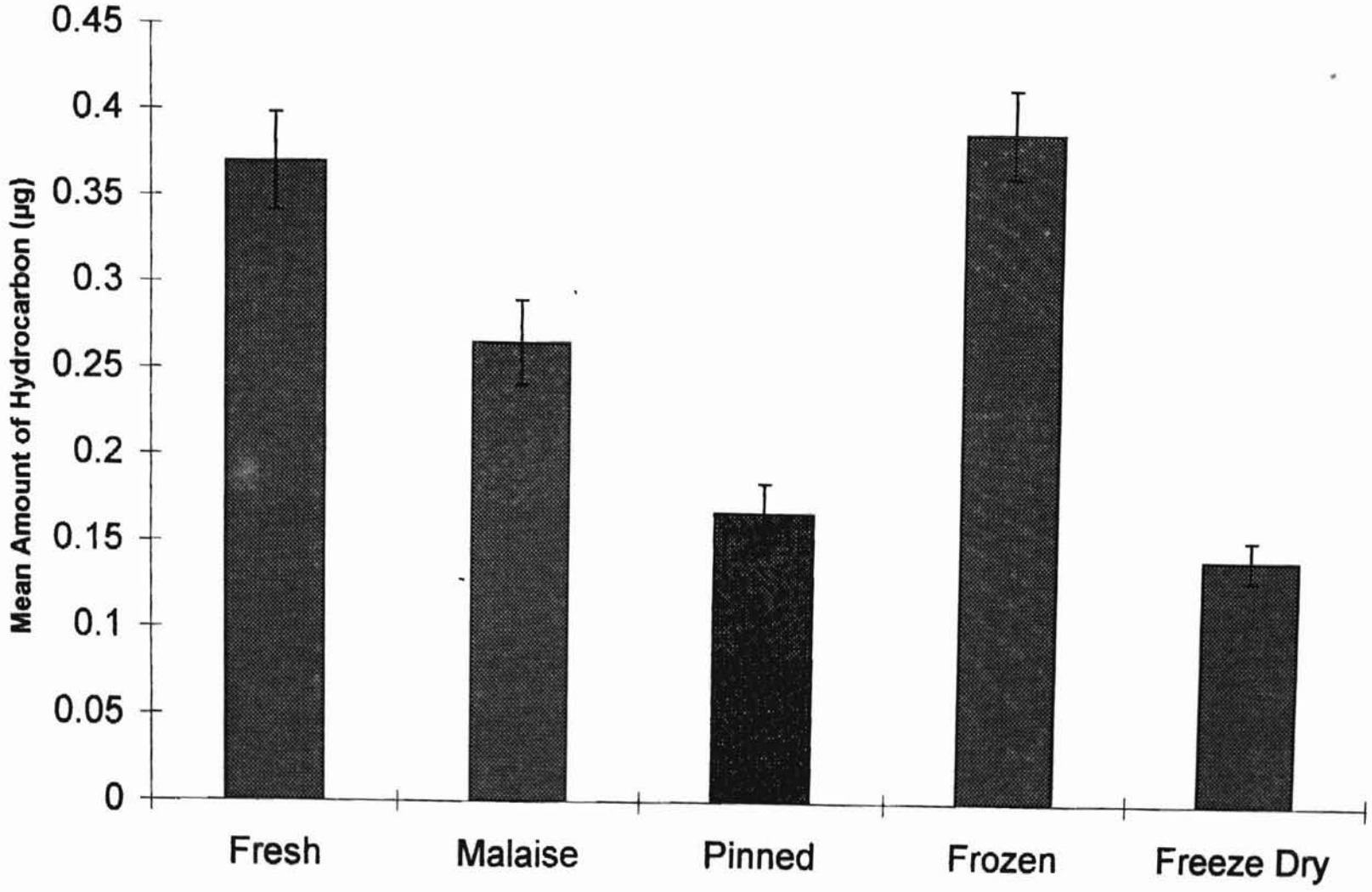
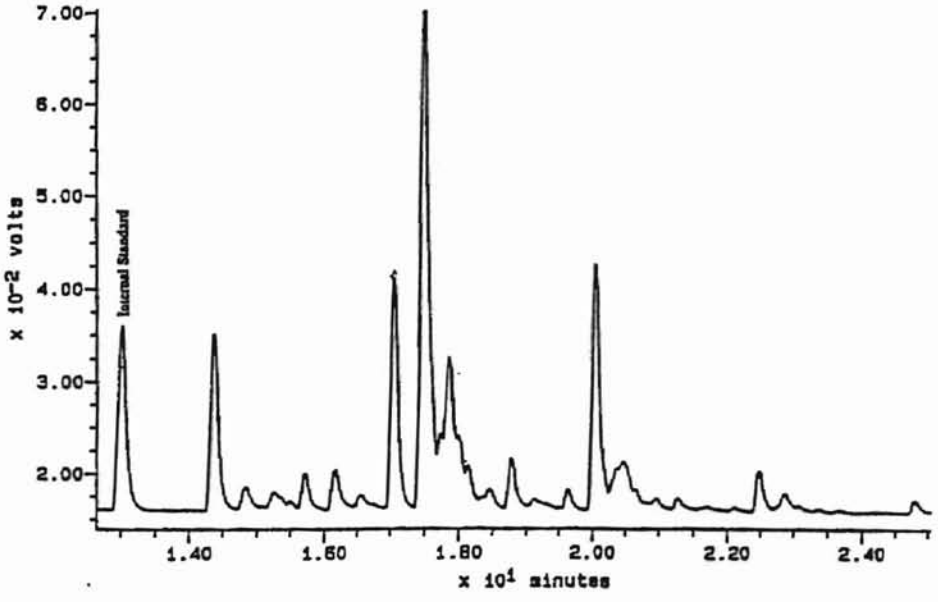

Mean Amount of Hydrocarbon (µg)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Fresh
Malaise
Pinned
Frozen
Freeze Dry

Fig. 14 Typical chromatogram of cuticular hydrocarbons from a freshly extracted female *T. mularis*.

Fig. 15 Typical chromatogram of cuticular hydrocarbons from a pinned (4 months) female *T. mularis*.


7.00
6.00
5.00
4.00
3.00
2.00
x 10⁻² volts
Internal Standard
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

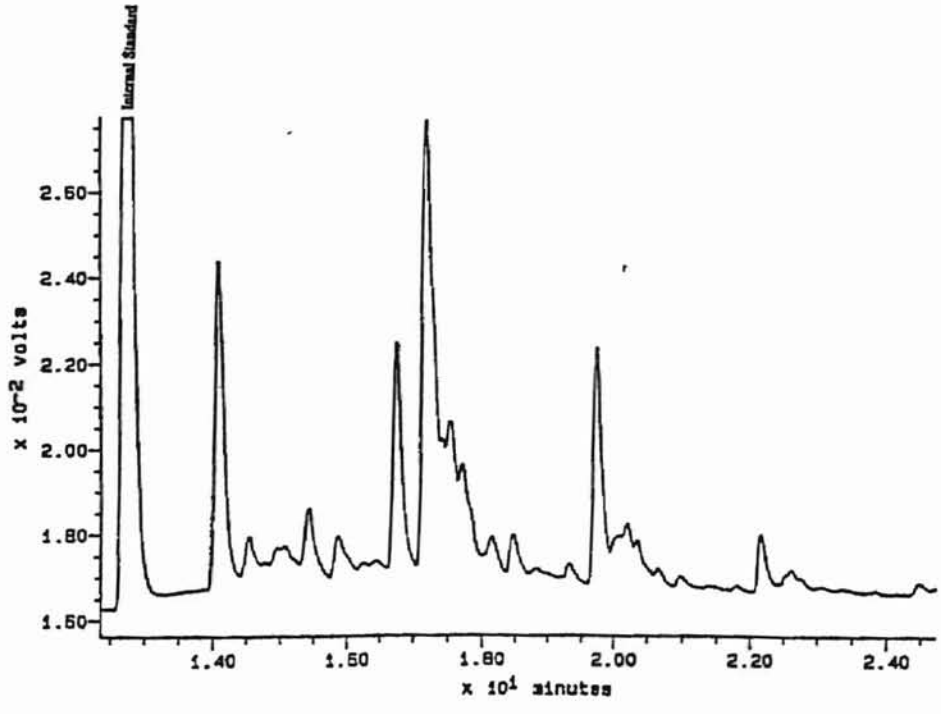
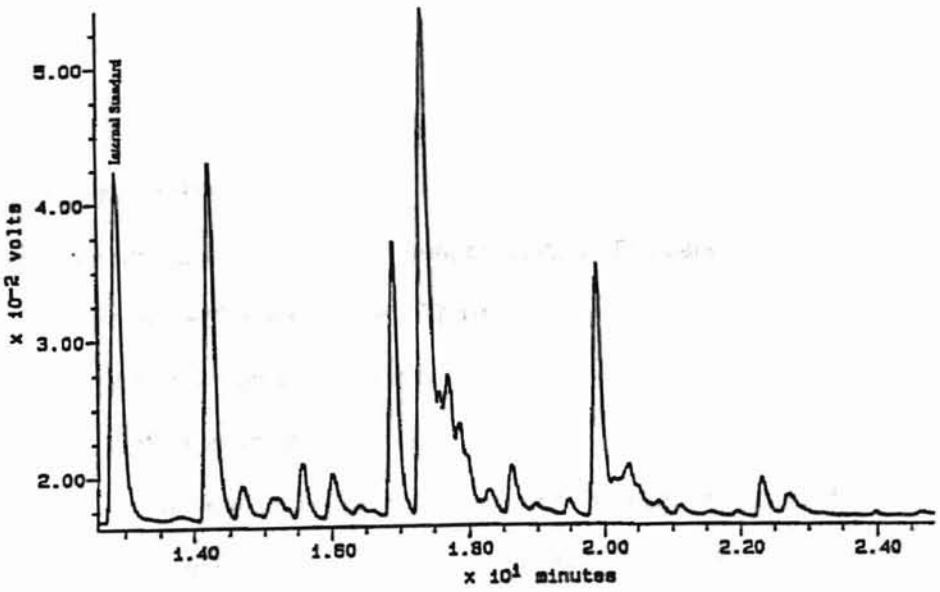
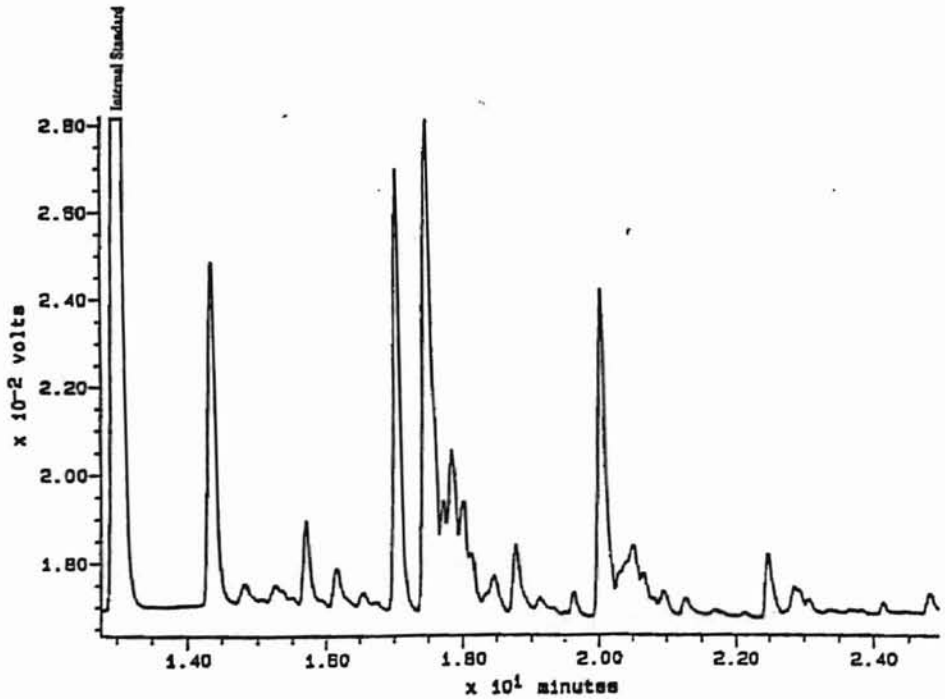

2.60
2.40
2.20
2.00
1.80
1.60
x 10⁻² volts
Internal Standard
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

Fig. 16 Typical chromatogram of cuticular hydrocarbons from a frozen (3 months) female *T. mularis.*

Fig. 17 Typical chromatogram of cuticular hydrocarbons from a freeze dried female *T. mularis.*


Internal Standard
5.00
4.00
3.00
2.00
x 10-2 volts
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes


Internal Standard
2.80
2.60
2.40
2.20
2.00
1.80
x 10-2 volts
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

of a freshly extracted fly. Furthermore, the chromatogram of a freeze-dried specimen resembled that of a pinned (4 months) specimen.

### Internal Hydrocarbons of *T. mularis*

Histograms in figure 18 represent the internal hydrocarbons extracted from *T. mularis* which had been processed under five different conditions. The bars represent means for the five groups of specimens (n=10 in each case). These results coincide with figure 13, in that the amount of internal hydrocarbons extracted from specimens frozen for 3 months resembled the amount of hydrocarbons extracted from fresh specimens.

In this case the amount of internal hydrocarbon extracted from pinned specimens (4 months) and freeze-dried specimens was approximately two times that of fresh flies and frozen flies. A possible explanation for this phenomenon was that the cuticular hydrocarbons were less easily extracted from pinned and freeze dried flies and did not dissolve in an external hexane extraction of the duration used in this study. Instead, they were extracted when the insect was homogenized and subjected to the Bligh and Dyer method of extraction.

Much more internal hydrocarbon is extracted from a pinned specimen than a freshly extracted specimen (Figures 19 & 20). The amount of internal hydrocarbons obtained from a lyophilized specimen was similar to that of a specimen which had been pinned (Figure 21). Also, the amount of internal hydrocarbons extracted from a frozen specimen (Figure 22) closely resembled that from a freshly extracted specimen (figure 20).

### Total Hydrocarbon Fractions of *T. mularis*

The histograms in figure 23 represent the total amount of cuticular hydrocarbons and internal hydrocarbons extracted from *T. mularis* which had been processed under five different conditions. The bars represent means for the five groups of specimens (n=10 in each case). It was apparent that the total amount of hydrocarbons obtained from *T. mularis* stored under different conditions was approximately the same for all storage conditions. Specimens pinned for 4 months yielded slightly higher amounts of total hydrocarbons, although they yielded lower amounts of cuticular hydrocarbons.

Fig. 18 Amounts of internal hydrocarbons extracted from *T. mularis* stored under different conditions.

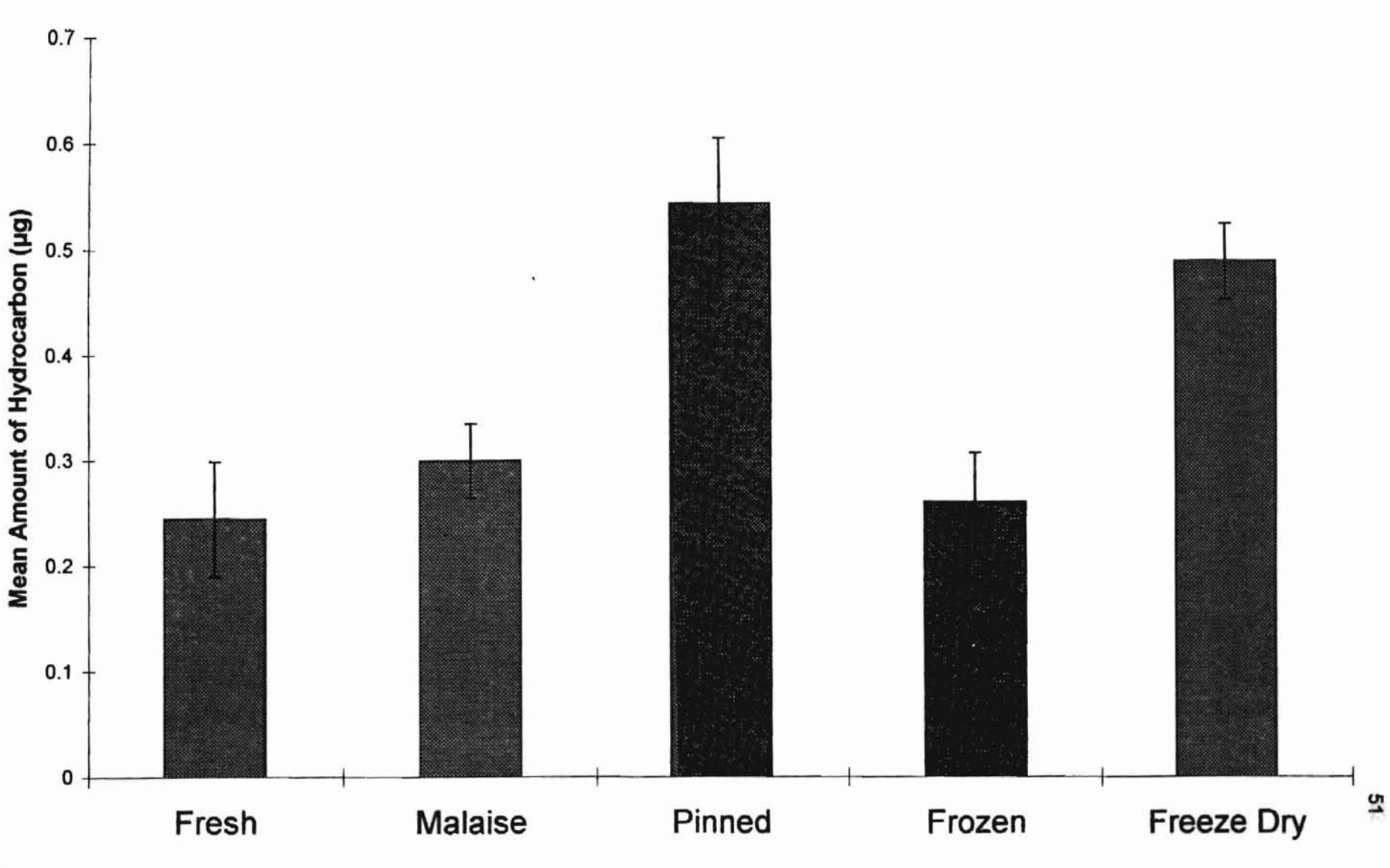
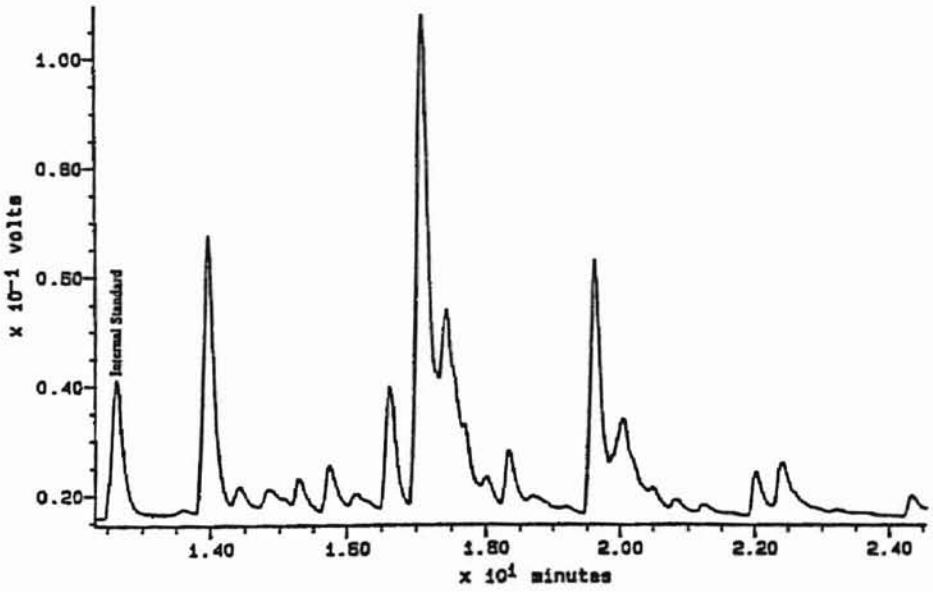

Mean Amount of Hydrocarbon (µg)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Fresh
Malaise
Pinned
Frozen
Freeze Dry

Fig. 19 Typical chromatogram of internal hydrocarbons from a pinned (4 months) female *T. mularis*.

Fig. 20 Typical chromatogram of internal hydrocarbons from a freshly extracted female *T. mularis*.


1.00
0.80
0.60
0.40
0.20
x 10⁻¹ volts
Internal Standard
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

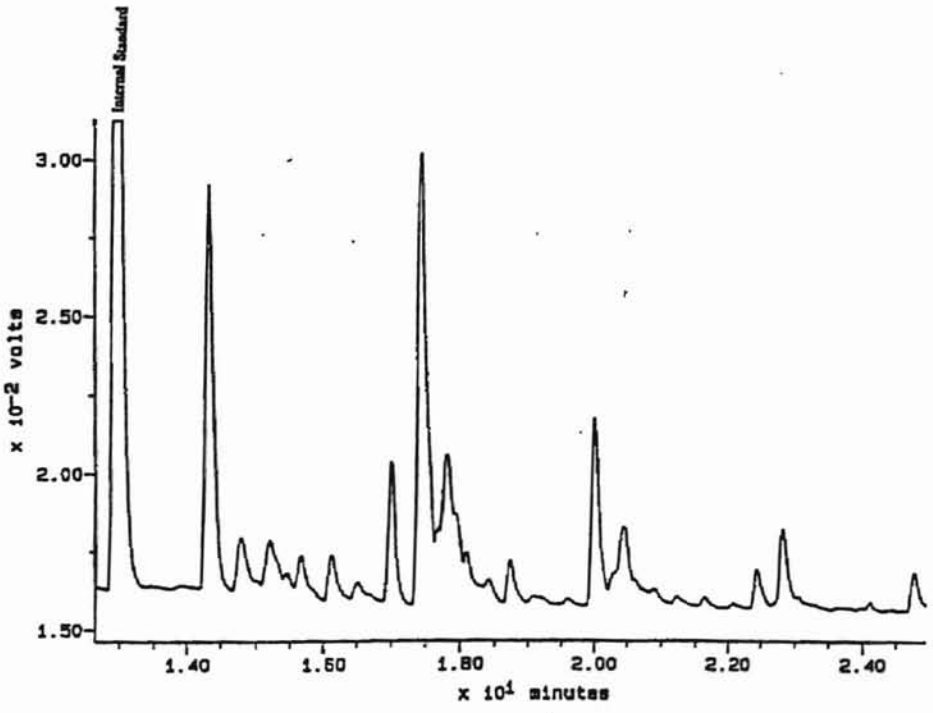
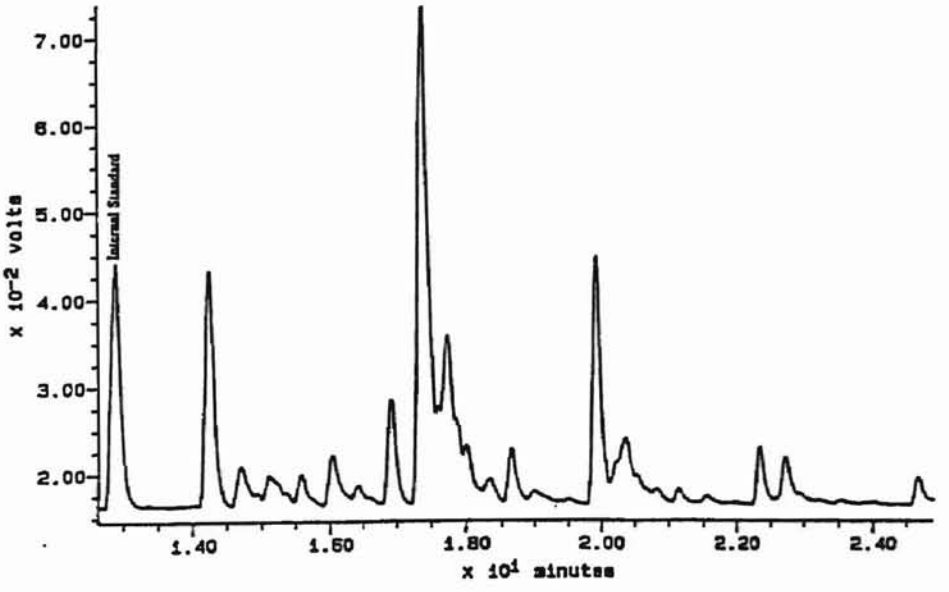

3.00
2.50
2.00
1.50
x 10⁻² volts
Internal Standard
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

Fig. 21 Typical chromatogram of internal hydrocarbons from a freeze dried female *T. mularis.*

Fig. 22 Typical chromatogram of internal hydrocarbons from a frozen (3 months) female *T. mularis.*


Internal Standard
7.00
6.00
5.00
4.00
3.00
2.00
x 10-2 volts
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

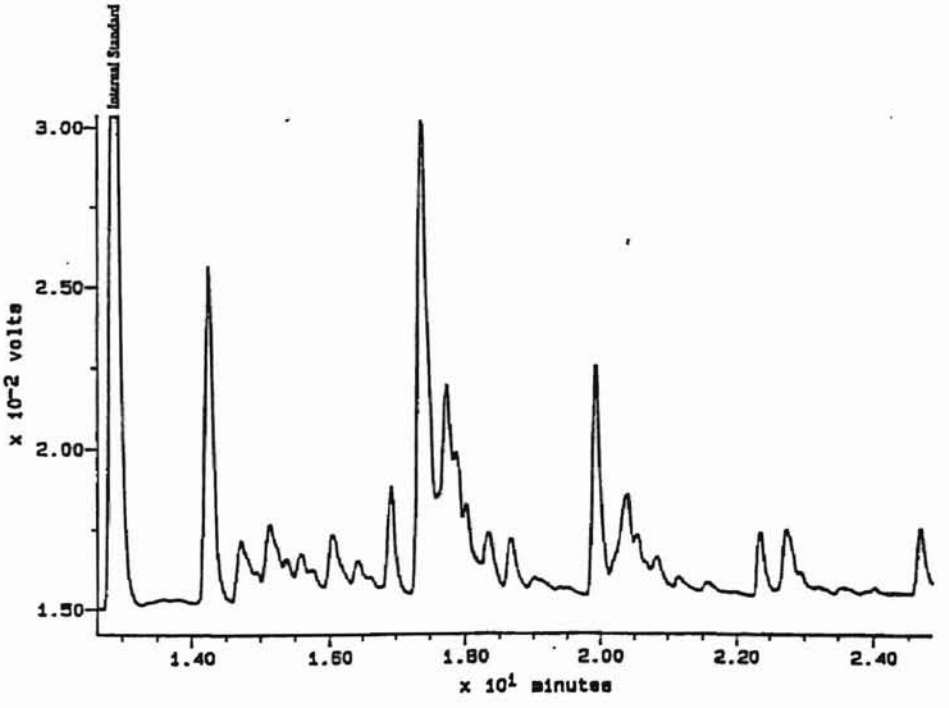
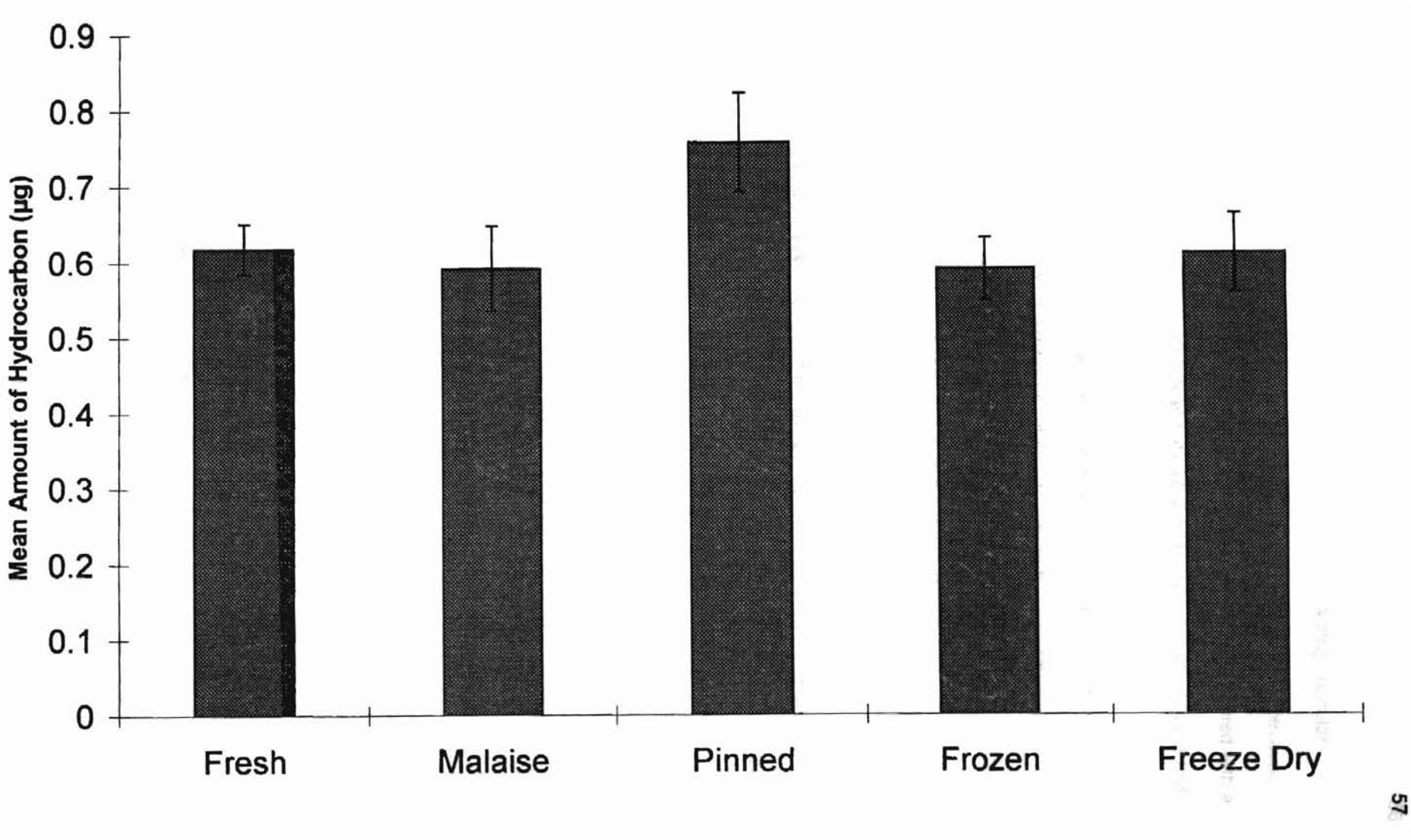

Internal Standard
3.00
2.50
2.00
1.50
x 10-2 volts
1.40
1.60
1.80
2.00
2.20
2.40
x 10¹ minutes

Fig. 23 Total amount of hydrocarbons extracted from *T. mularis* stored under different conditions.


Mean Amount of Hydrocarbon (µg)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Fresh
Malaise
Pinned
Frozen
Freeze Dry

As stated previously, it was originally assumed that when extracting cuticular hydrocarbons from fresh or frozen *T. mularis*, more hydrocarbons were obtained because internal hydrocarbons were being extracted. However, when frozen flies were treated with a series of 30-second extractions, nearly all cuticular hydrocarbons were obtained in the first extraction and virtually none were obtained in subsequent extractions (Figures 24 & 25). Somewhat different results were attained with pinned specimens in that less cuticular hydrocarbon was obtained in the first extraction relative to amounts in subsequent extractions (Figures 26 & 27). These results support a hypothesis that cuticular hydrocarbons continuously leach out of pinned specimens when exposed to multiple hexane extractions.

In addition, there were internal lipids extracted in all five hexane washings from the pinned specimen (fig. 26) which probably resulted from hexane seeping into the insect through cracks in the exoskeleton. This means that the increased amounts of hydrocarbons in fresh and frozen flies could not be attributed to acquiring internal hydrocarbons. If this were the case, then there would have been elevated levels of hydrocarbons in pinned specimens. Thus, the hypothesis that freshly extracted and frozen *T. mularis* yielded more hydrocarbons due to internal lipid extraction was discounted.

In pinned *T. mularis* the hydrocarbons leach out into the solvent over the course of extractions. Brill and Bertsch (1985) found similar results when extracting frozen worker ants of *Solenoptes richteri* Forel (the black imported fire ant) in Alabama. They attributed the recovery of hydrocarbons upon second and third solvent washings as a diffusion problem. The cuticular hydrocarbons are contained in the epicuticular matrix, which contains wax, sterol esters, alcohols and free fatty acids in addition to the hydrocarbons. In order to extract the hydrocarbons, they must be removed from the matrix and into the bulk of the solvent. For this to occur, solvent molecules must penetrate the lipid matrix, or the matrix itself must be broken down. A plausible explanation for this phenomenon was that the cuticular hydrocarbons were somehow embedded in the wax layer of the cuticle of pinned and freeze dried flies and did not leach out easily in an external hexane washing. Instead, the hydrocarbons were liberated when the insect was homogenized and subjected to Bligh and Dyer extraction.

Fig. 24 High performance thin layer chromatography plate of lipid extract from multiple extractions of a single, frozen *T. mularis*.

Lane 1. Standard containing cholesterol (C), free fatty acid (FFA), monoglyceride (MG), diglyceride (DG), triglyceride (TG), sterol ester (SE), cholesterol ester (CE), and hydrocarbon (HC).

Lane 2. First 30 second hexane extraction.

Lane 3. Second 30 second hexane extraction.

Lane 4. Third 30 second hexane extraction.

Lane 5. Fourth 30 second hexane extraction.

Lane 6. Fifth 30 second hexane extraction.

Lane 7. Standard containing primary alcohol ($1^0$ ALC), cholesterol ester (CE), and hydrocarbon (HC).

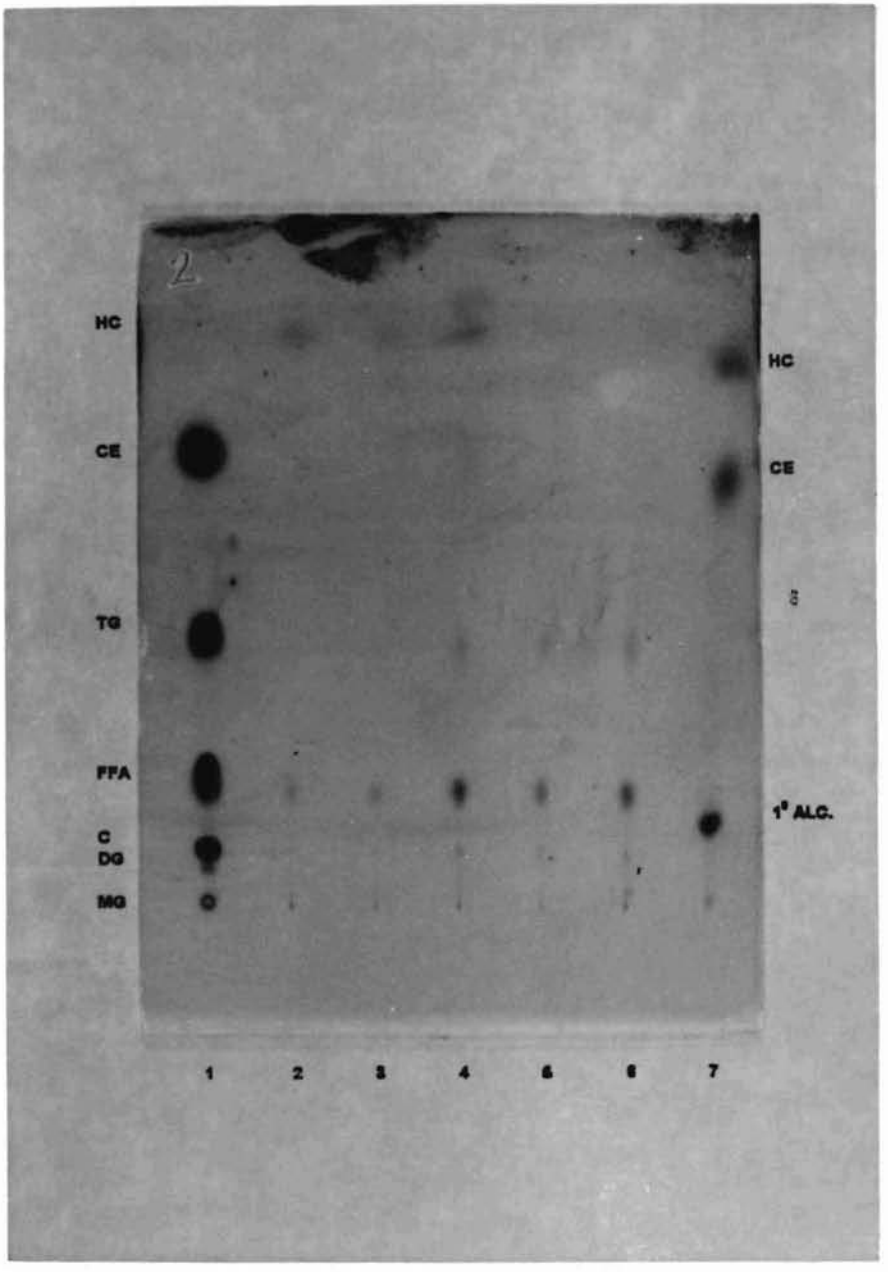

2
HC
CE
TG
FFA
C
DG
MG
HC
CE
1° ALC.
1
2
3
4
5
6
7

Fig. 25 Amount of hydrocarbons obtained from five hexane extractions of the same *T. mularis* which had been frozen 3 months.

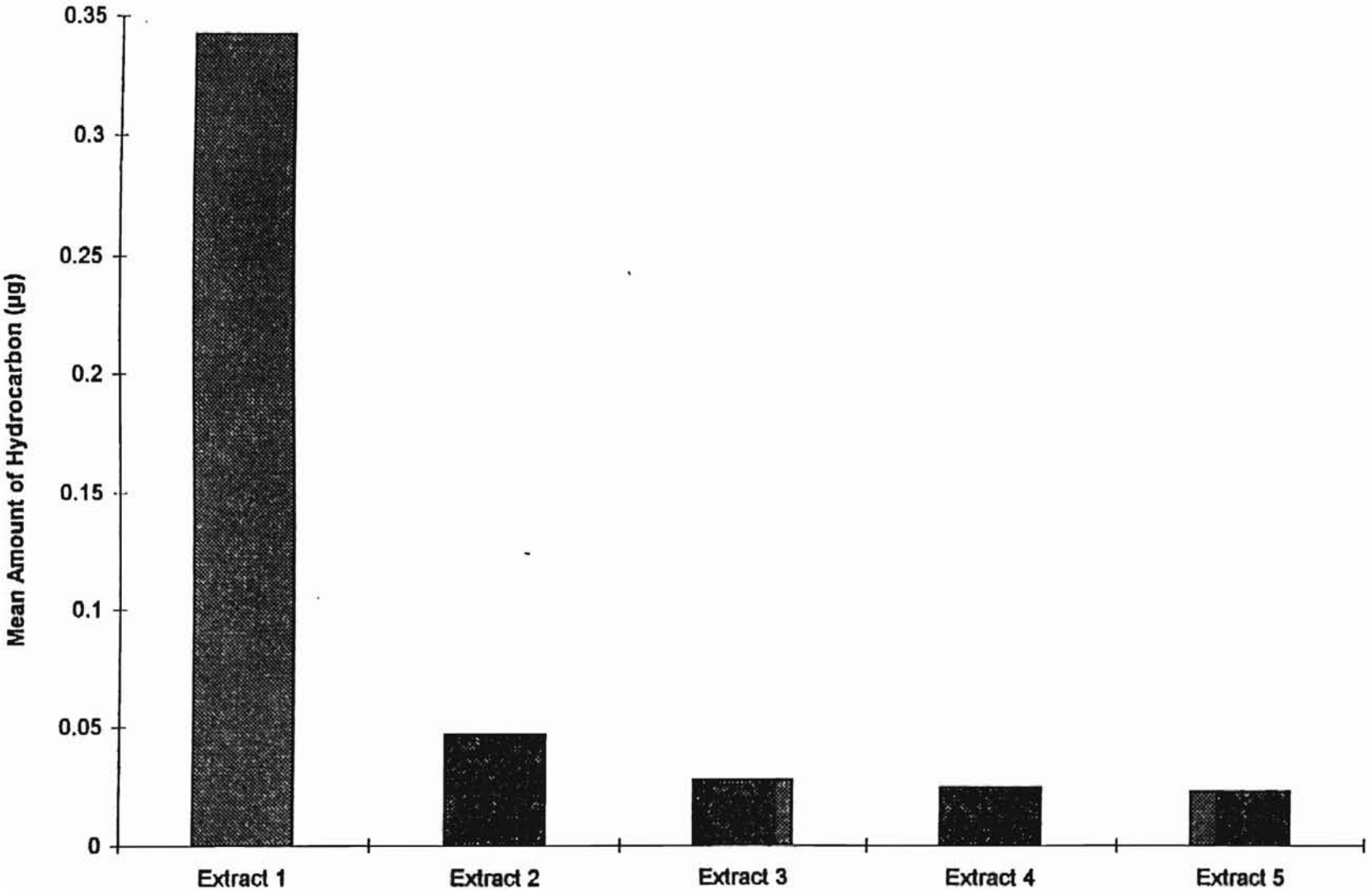

Mean Amount of Hydrocarbon (µg)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Extract 1
Extract 2
Extract 3
Extract 4
Extract 5

Fig. 26 High performance thin layer chromatography plate of lipid extract from multiple extractions of a single, pinned *T. mularis*.

Lane 1. Standard containing cholesterol (C), free fatty acid (FFA), monoglyceride (MG), diglyceride (DG), triglyceride (TG), sterol ester (SE), cholesterol ester (CE), and hydrocarbon (HC).

Lane 2. First 30 second hexane extraction.

Lane 3. Second 30 second hexane extraction.

Lane 4. Third 30 second hexane extraction.

Lane 5. Fourth 30 second hexane extraction.

Lane 6. Fifth 30 second hexane extraction.

Lane 7. Standard containing primary alcohol ($1^0$ ALC), cholesterol ester (CE), and hydrocarbon (HC).

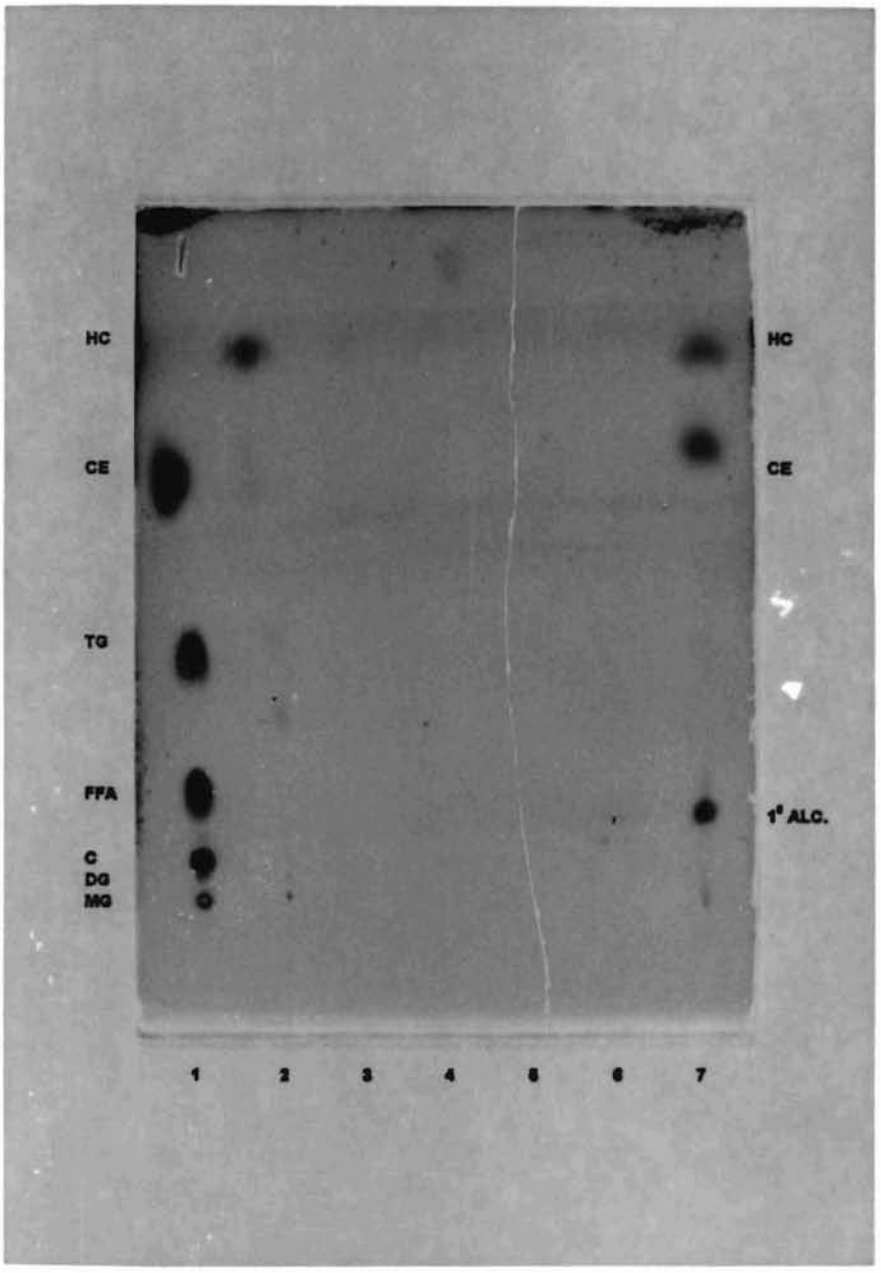
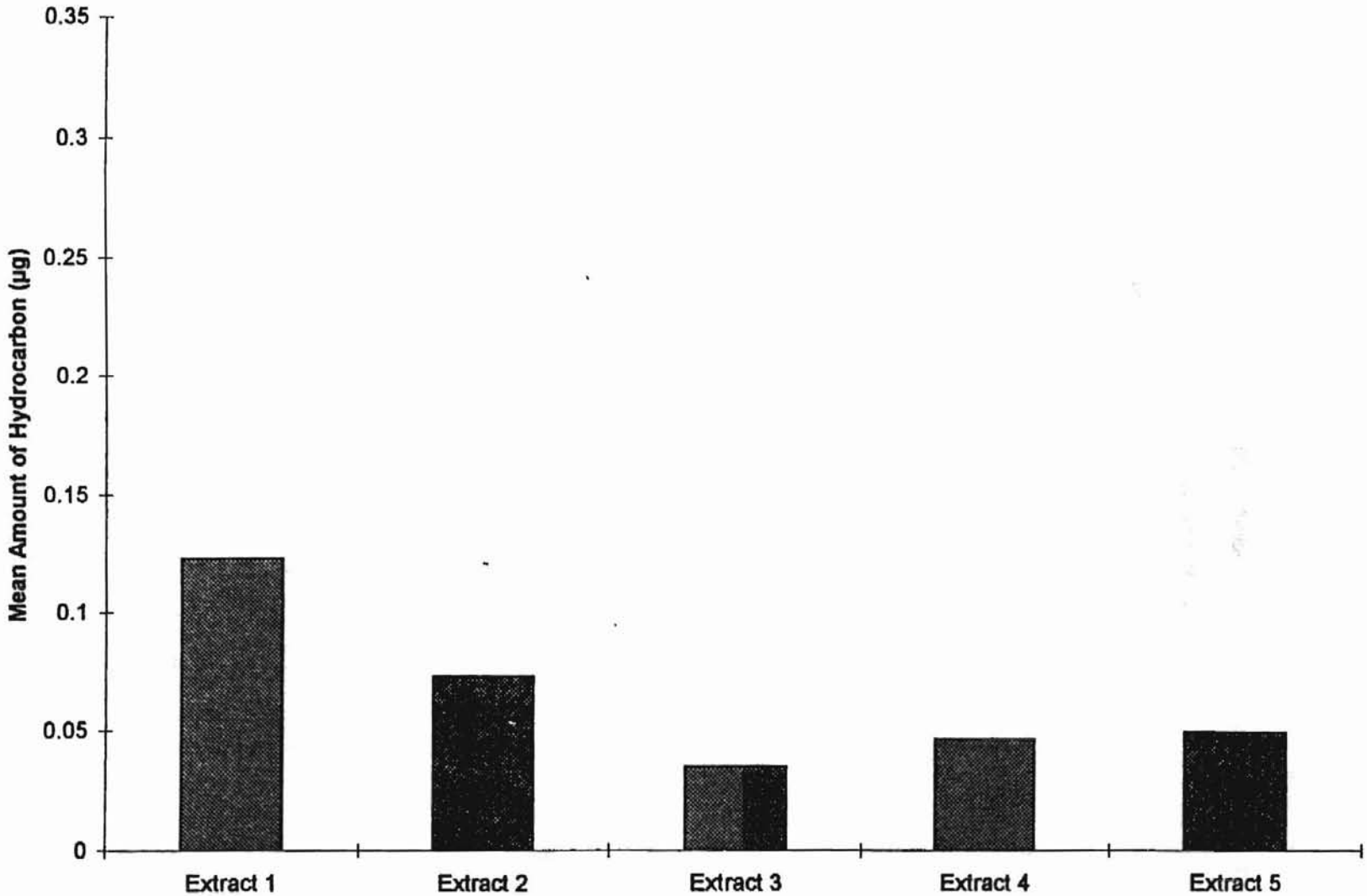

HC
CE
TG
FFA
C
DG
MG
HC
CE
1° ALC.
1
2
3
4
5
6
7

Fig. 27 Amount of hydrocarbons obtained from five hexane extractions of the same *T. mularis* which had been pinned 4 months.


Mean Amount of Hydrocarbon (µg)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Extract 1
Extract 2
Extract 3
Extract 4
Extract 5

Canonical Discriminant Analysis of Cuticular Hydrocarbons

Figure 28 depicts a two-dimensional canonical analysis of the cuticular hydrocarbons of female *T. mularis* which had been subjected to five storage treatments. Those conditions were freshly extracted specimens (chilled to immobilize), *T. mularis* collected in a malaise trap, *T. mularis* pinned for 4 months, *T. mularis* frozen for 3 months (these specimens were allowed to come to room temperature prior to extraction), and *T. mularis* which had been freeze dried. When external hydrocarbons from the treatments were compared by general linear models procedure (SAS Institute Inc. 1989) there were significant differences between groups in the MANOVA Wilks' Lambda test (F= 3.0991; DF=44; Pr>F 0.0001). When the discriminant analysis was executed, the program correctly identified 76% of the specimens. There were no misidentifications in the group of *T. mularis* from the malaise trap. The program misidentified one fresh *T. mularis* as a malaise trap specimen, one as a frozen, and one as lyophilized. Also, one pinned *T. mularis* was misidentified as lyophilized, while one frozen *T. mularis* was misidentified as fresh, and three frozen *T. mularis* as lyophilized specimens. Among freeze dried *T. mularis*, one was misidentified as fresh, one as a frozen, and two as pinned specimens.

Improved discrimination of fresh, pinned and freeze dried *T. mularis* was obtained when malaise and frozen groups were dropped from the analysis (Figure 29). Pinned specimens were separated from freshly extracted specimens and lyophilized specimens along canonical axis 1, while canonical axis 2 separated the fresh and lyophilized specimens. In this analysis there were significant differences between groups in the MANOVA Wilks' Lambda test (F= 22.8718; DF=24; Pr>F 0.0001). When the discriminant analysis was executed this time the program correctly identified 96% of the flies. There was one misidentification where a freeze-dried *T. mularis* was categorized with the freshly extracted specimens.

When fresh *T. mularis* were compared only against pinned *T. mularis* there were also significant differences between groups in the MANOVA Wilks' Lambda test (F= 8.1738; DF=12; Pr>F 0.005), and there were no misidentifications in the discriminant analysis. When fresh *T. mularis* were compared only against freeze dried *T. mularis* there were significant differences

Fig. 28 Canonical discriminant analysis of cuticular hydrocarbons from *T. mularis* processed under five different conditions.

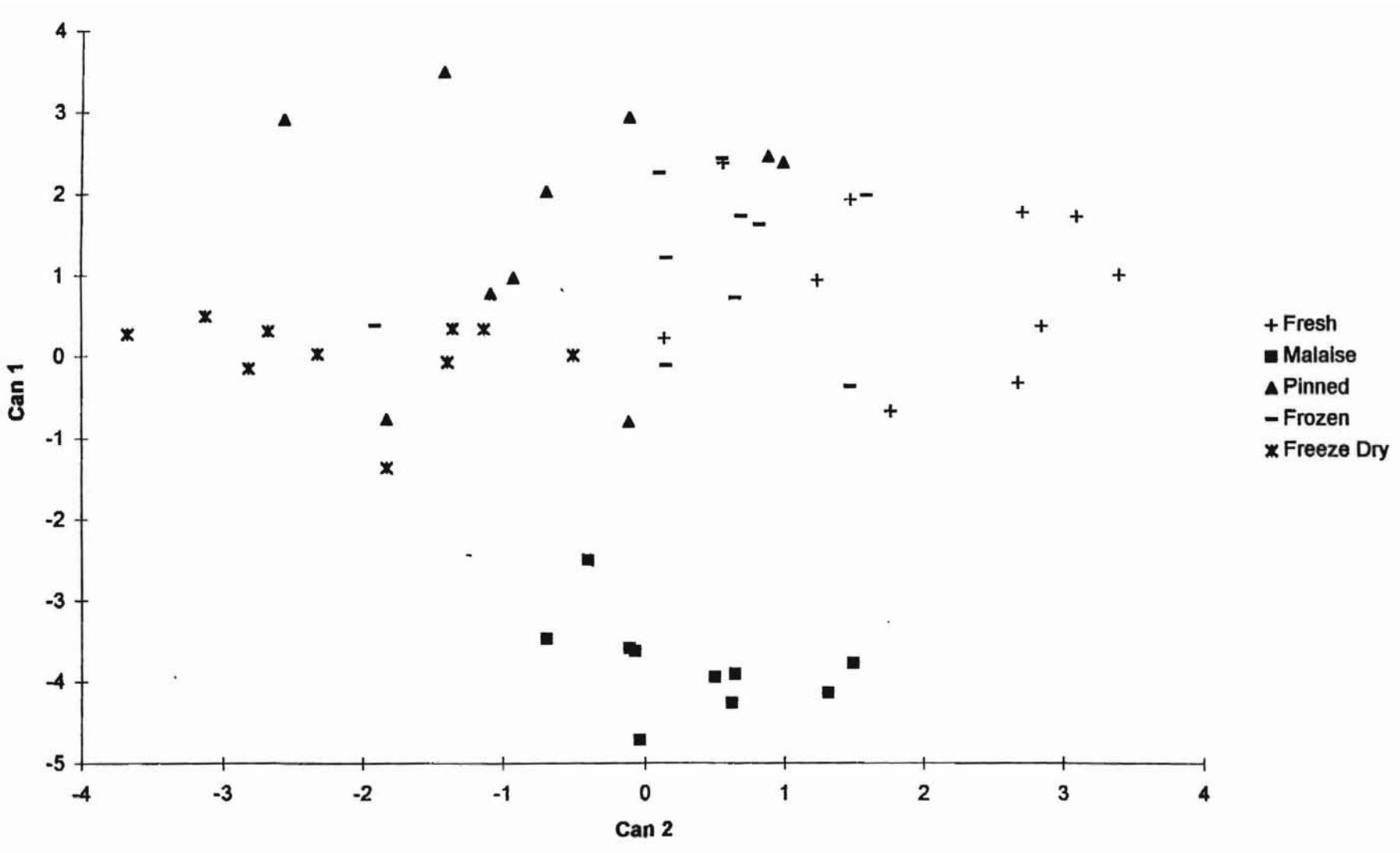
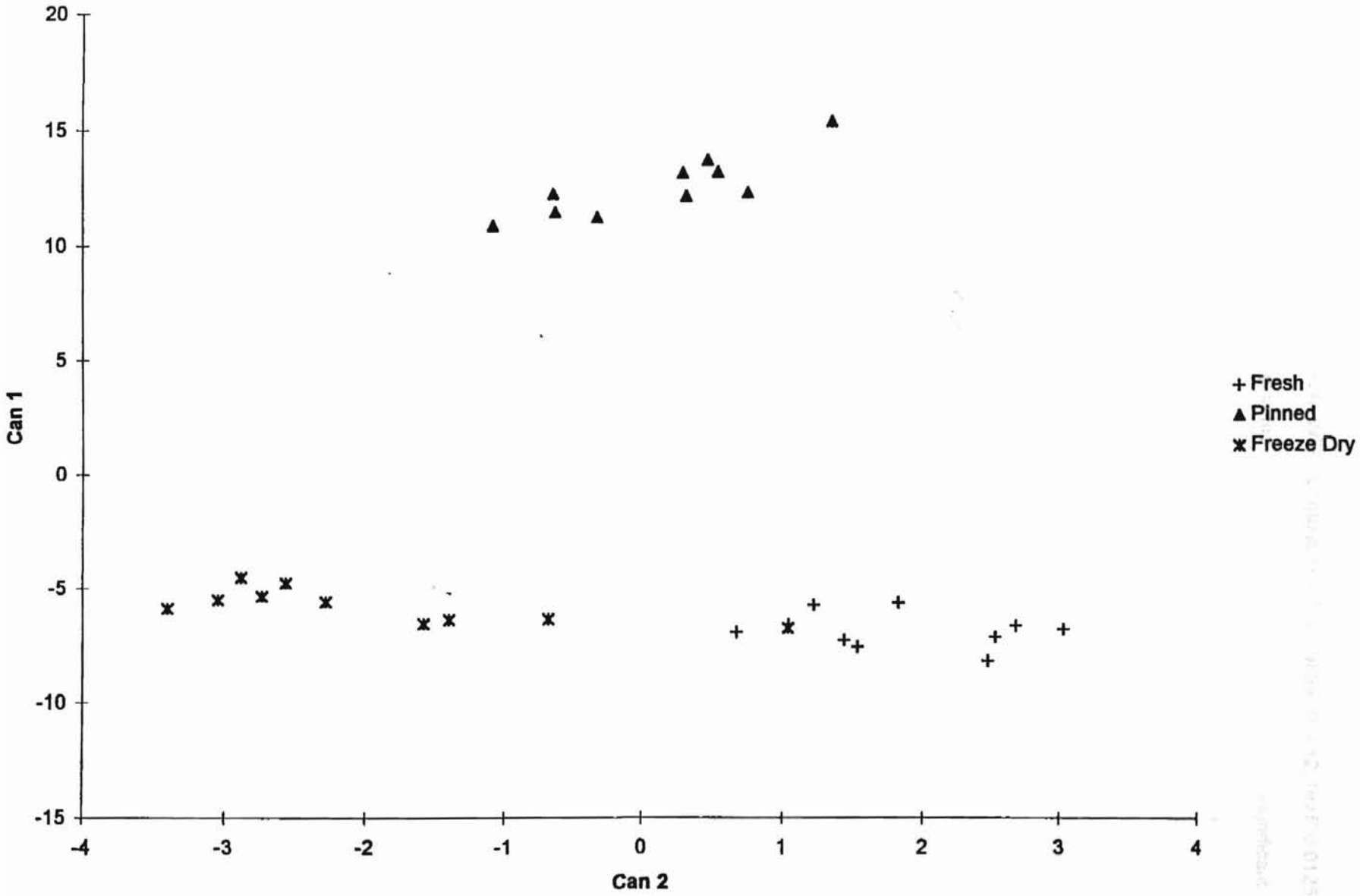

Can 1
Can 2
4
3
2
1
0
-1
-2
-3
-4
-5
-4
-3
-2
-1
0
1
2
3
4
+ Fresh
■ Malaise
▲ Pinned
– Frozen
✱ Freeze Dry

Fig. 29 Canonical discriminant analysis of cuticular hydrocarbons from *T. mularis* processed under three different conditions.


20
15
10
5
0
-5
-10
-15
Can 1
-4
-3
-2
-1
0
1
2
3
4
Can 2
+ Fresh
▲ Pinned
✱ Freeze Dry

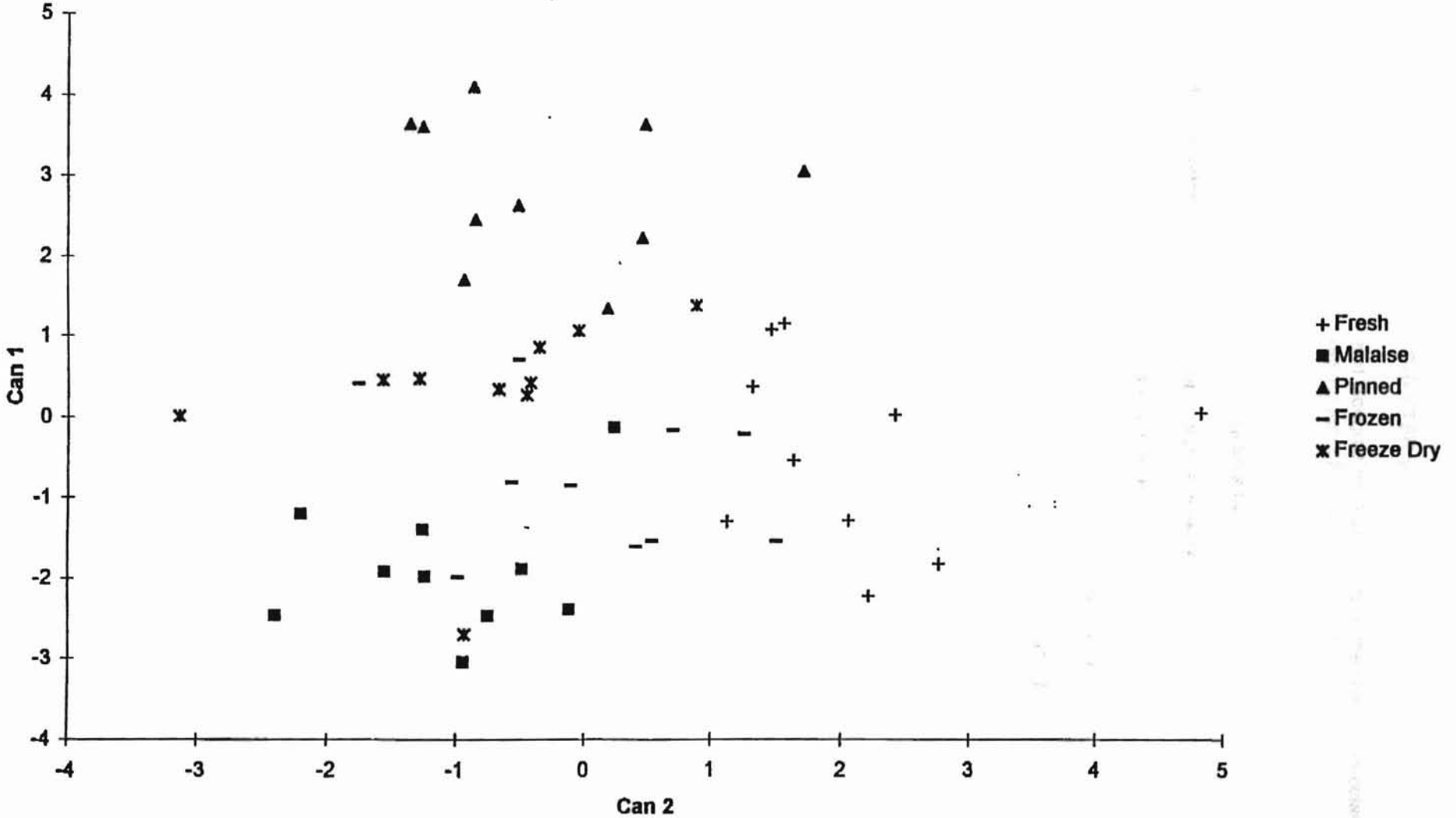
between classes as well in the MANOVA Wilks' Lambda test (F= 5.9899; DF=12; Pr>F 0.0125) and no misidentifications of specimens in the discriminant analysis. There were no significant differences between cuticular hydrocarbons of freshly extracted and frozen ( 3 months) *T. mularis*.

Canonical Discriminant Analysis of Internal Hydrocarbons of *T. mularis*

Figure 30 depicts a two-dimensional canonical analysis of the internal hydrocarbons of female *T. mularis* which had been subjected to five storage treatments. When internal hydrocarbons were compared among treatments by general linear models procedure (SAS Institute Inc. 1989) there were significant differences in the MANOVA Wilks' Lambda test (F= 3.2282; DF=48; Pr>F 0.0001). When the discriminant analysis was executed, the program correctly identified 84% of the flies. The program misidentified two fresh *T. mularis* as frozen specimens and one collected by malaise trap as a fresh specimen. Also one pinned *T. mularis* was categorized a freeze dried specimen. Among frozen specimens, one was misidentified as a fresh, one as caught by malaise trap, and one as freeze dried. One freeze dried *T. mularis* was misidentified as frozen.

These experiments show that there were statistically significant and reproducible differences in the composition of cuticular hydrocarbons which were extracted from female T. mularis stored under different conditions using hexane as the extracting solvent. More cuticular hydrocarbons were obtained from the flies when fresh or frozen than from other treatments.

Fig. 30 Canonical discriminant analysis of internal hydrocarbons from *T. mularis* processed under five different conditions.


5
4
3
2
1
0
-1
-2
-3
-4
Can 1
-4
-3
-2
-1
0
1
2
3
4
5
Can 2
+ Fresh
■ Malaise
▲ Pinned
– Frozen
✱ Freeze Dry

## CHAPTER V

### The Effects of Seasonal Distribution and Geographic Distribution on the Use of Cuticular Hydrocarbon Analysis for Identification of *T. mularis*

### Introduction

Results of the experiments described in chapters two and three indicated that the conditions under which the specimens of *T. mularis* were collected and stored influenced the use of cuticular hydrocarbon analysis to differentiate among them. These studies also indicated that the reliability of this technique might be influenced by age of flies or temporal distribution through the summer as well as differences in geographical regions of the state from which specimens are collected. Therefore, studies were designed to determine if there were differences in the cuticular hydrocarbon profiles of *T. mularis* collected at the same site throughout the season and if the profiles were different in those specimens collected from several geographic regions of the state.

### Methods and Materials

#### Experiment 1

This study was designed to determine if the cuticular hydrocarbon profiles were different in *T. mularis* collected at the same site over the season. In order to minimize variability all specimens used in this study were collected fresh (off cattle). Ten *T. mularis* were collected at CTER pasture two at weekly intervals from 8 June through 1 September 1995 (15 collections). Specimens were collected in clean 7ml scintillation vials which had been pre-rinsed with hexane. They were then chilled on ice to immobilize them and were returned to the laboratory where they were extracted with hexane to remove cuticular hydrocarbons while still alive.

#### Experiment 2

This study was designed to determine if there was a difference in the cuticular hydrocarbon profiles of *T. mularis* from different areas of Oklahoma. Female *T. mularis* were collected in malaise traps during the summer of 1995 from Lincoln, Muskogee, McCurtain and Wagoner counties in Oklahoma. Traps were operated for approximately 24 hours. Specimens were placed on ice as soon as they were removed from the trap top and returned to the

laboratory for cuticular hydrocarbon extraction. Cuticular hydrocarbon extraction and analysis of the hydrocarbon data was performed in the same manner as described in the previous chapters.

## Results and Discussion

Figure 31 shows a two-dimensional canonical discriminant analysis of the cuticular hydrocarbons of *T. mularis* collected from CTER pasture two during the summer of 1995. A comparison of cuticular hydrocarbons from specimens collected on different dates by general linear models procedure (SAS Institute Inc. 1989), indicates there were significant differences between groups in the MANOVA Wilks' Lambda test (F= 2.0563; DF=168; Pr>F 0.0001). A better indication of the differences are presented if only the group centroids are plotted, Figure 32. The graph of the centroids separate the collection dates into four areas. The first group of centroids consists of specimens collected from 8 June through 5 July 1995 at the beginning of the first seasonal emergence of this species. A second cluster of centroids includes the specimens collected from 12 July through 25 July 1995. The third cluster of centroids includes the specimens collected from 5 August through 25 September 1995. Specimens collected at the end of the season on 1 September 1995 were extreme outliers from the rest of the clusters.

These differences appear to reflect real changes in the cuticular hydrocarbon composition of the specimens in the field over the season. It is not known whether these changes have a functional significance. A possible explanation for this phenomenon over a season would be that there are different emergence patterns of the *T. mularis* at CTER pasture two and that a change in the larval food supply might be reflected in subtle differences in hydrocarbon patterns as adults. Lecuona *et al.* (1991) reported that fungal spore germination of *Beauveria bassiana* (Balsamo) Vuillemin or *B. brongniartii* (Saccardo) Petch caused alterations of the cuticular hydrocarbons of *Ostrinia nubilalis* (Hubner) and *Melolontha melolontha* L. Logic dictates that fungal infections could be a plausible explanation for changes in the hydrocarbons of *T. mularis* in a season. Little is known about the biology of this species, especially the larval stage, and changes in larval habitat or food sources may be reflected in slightly different cuticular hydrocarbon patterns of adults.

Fig. 31 Canonical discriminant analysis of cuticular hydrocarbons collected from fresh T. mularis during the summer of 1995 at CTER pasture two.

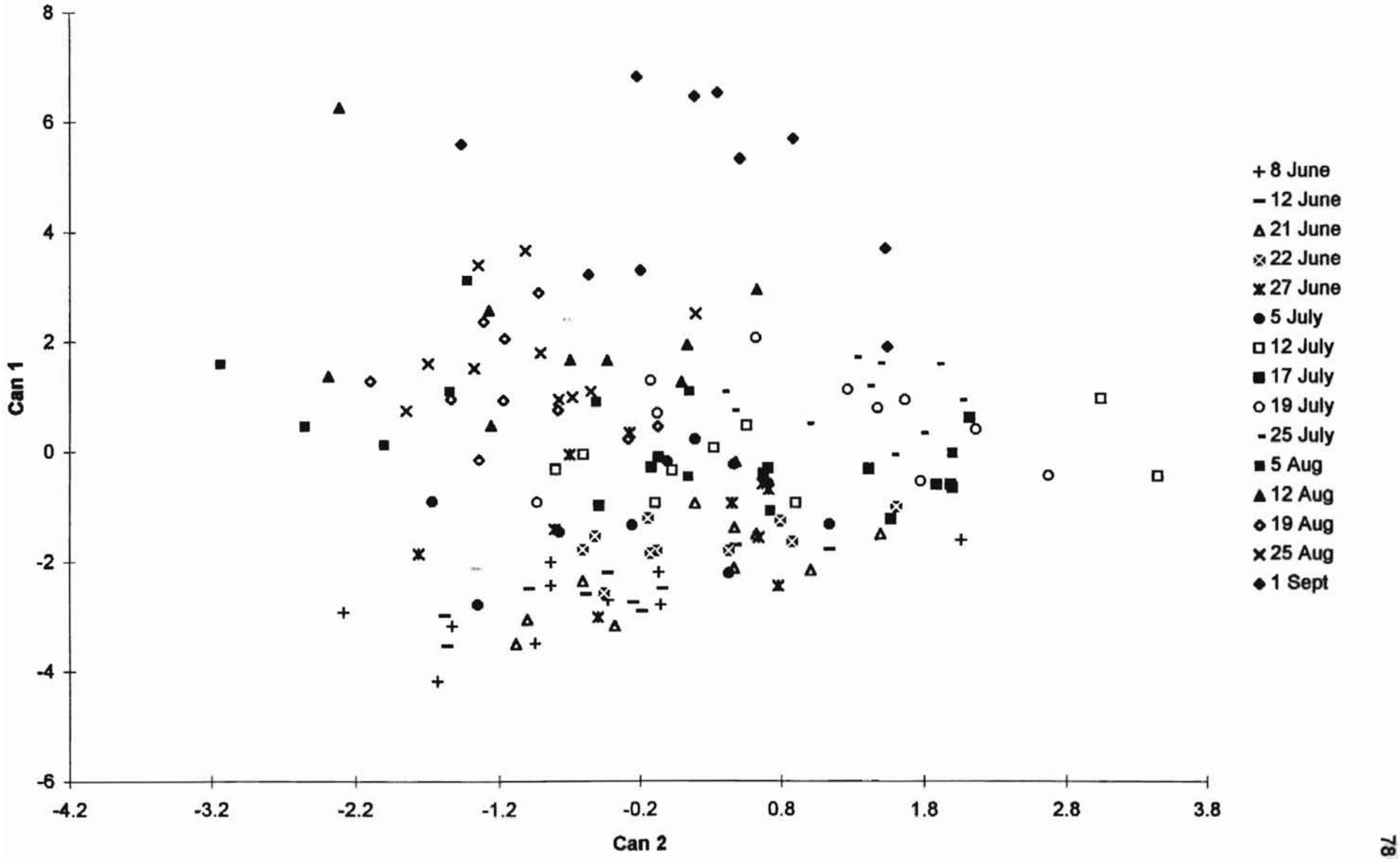
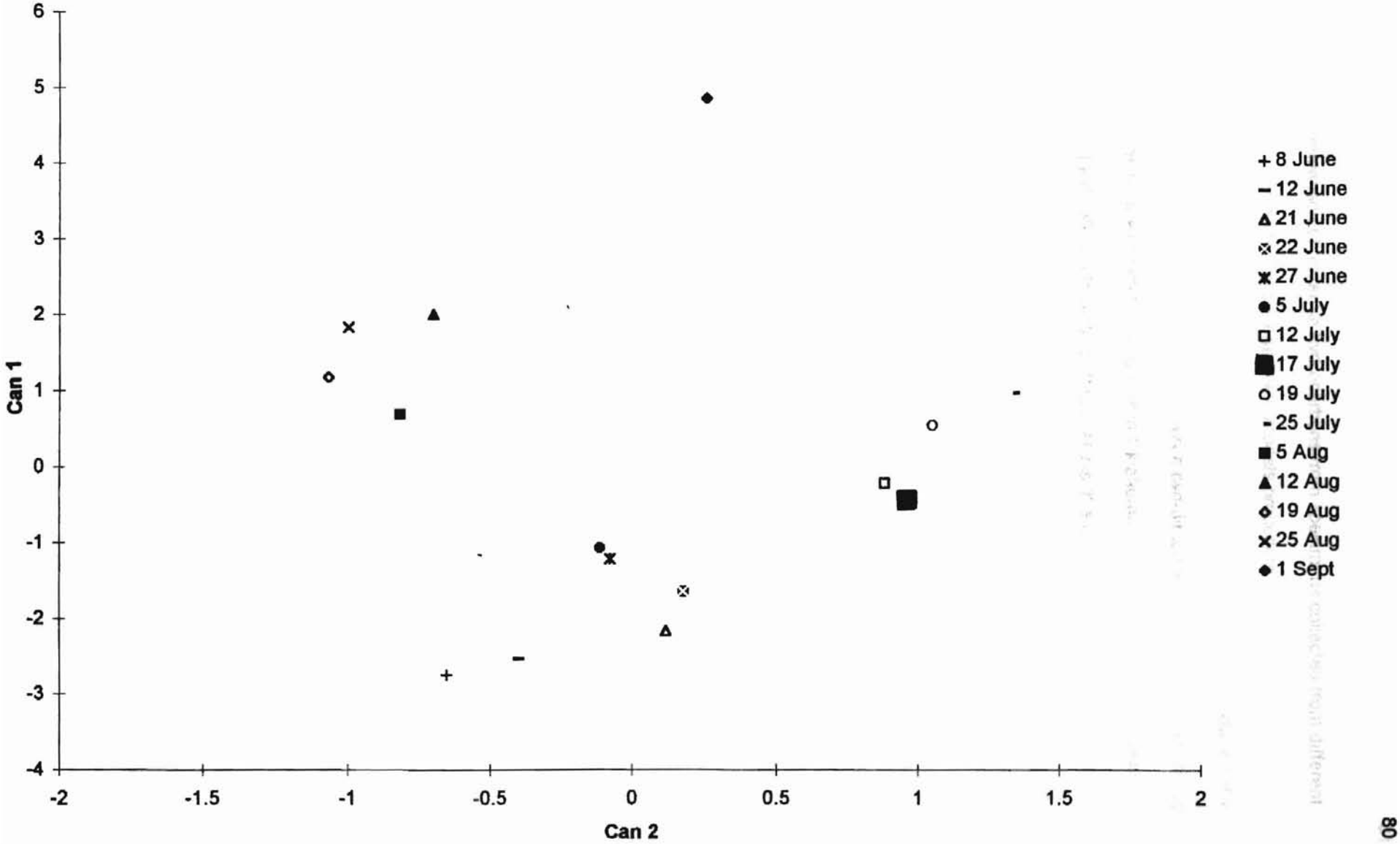
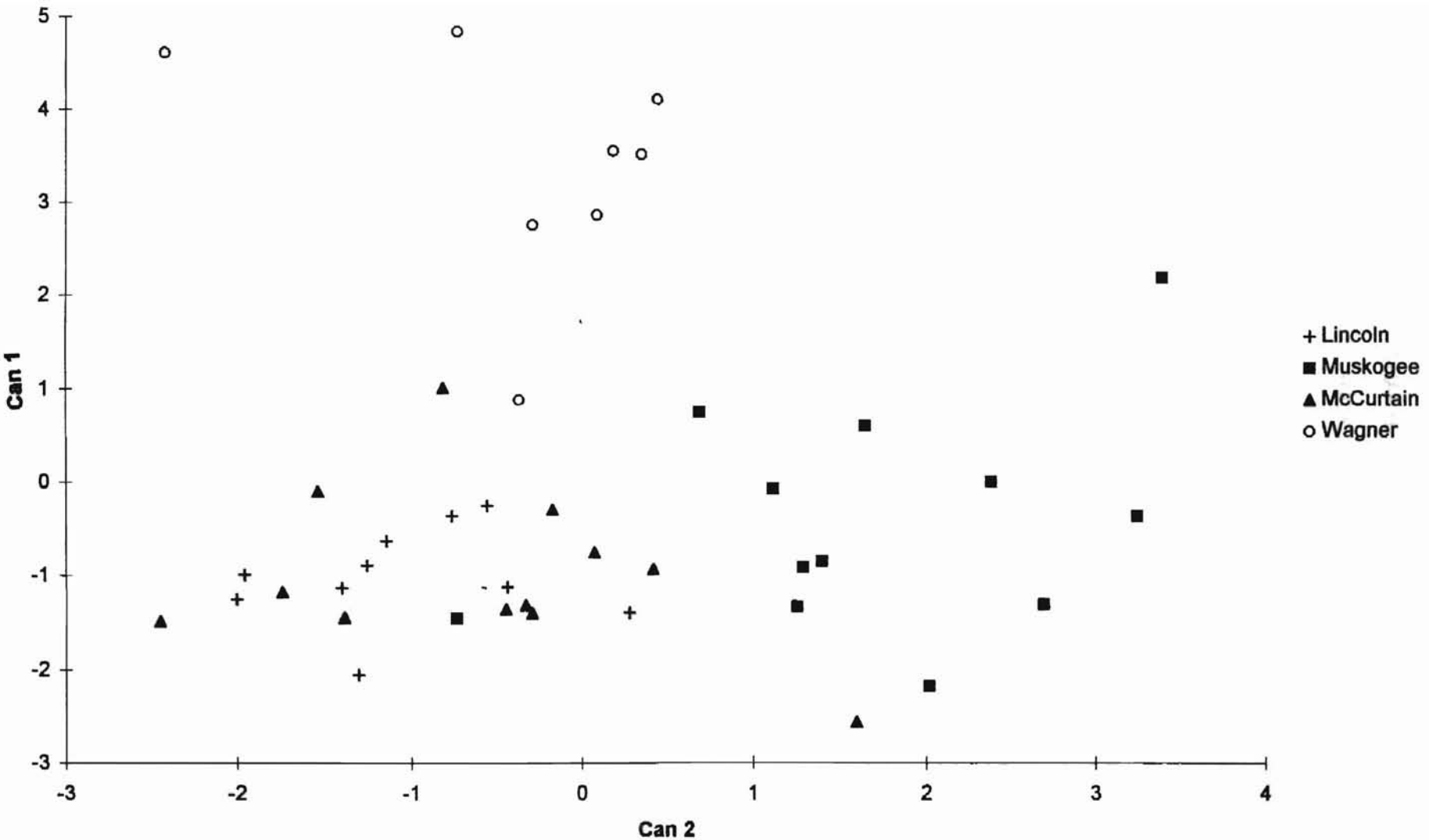

8
6
4
2
0
-2
-4
-6
Can 1
-4.2
-3.2
-2.2
-1.2
-0.2
0.8
1.8
2.8
3.8
Can 2
+ 8 June
– 12 June
△ 21 June
⊗ 22 June
✱ 27 June
● 5 July
□ 12 July
■ 17 July
○ 19 July
- 25 July
■ 5 Aug
▲ 12 Aug
◇ 19 Aug
× 25 Aug
◆ 1 Sept

Fig. 32 Canonical discriminant analysis of cuticular hydrocarbons collected from fresh *T. mularis* during the summer of 1995 at CTER pasture two. Showing group centroids (canonical mean values for groups).


Can 1
Can 2
6
5
4
3
2
1
0
-1
-2
-3
-4
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
+ 8 June
– 12 June
▲ 21 June
⊠ 22 June
✱ 27 June
● 5 July
□ 12 July
■ 17 July
○ 19 July
- 25 July
■ 5 Aug
▲ 12 Aug
◇ 19 Aug
× 25 Aug
◆ 1 Sept

A comparison of external hydrocarbons from specimens collected from different counties and analyzed by the general linear models procedure (SAS Institute Inc. 1989), indicated significant differences between groups in the MANOVA Wilks' Lambda test (F= 2.4443; DF=36; Pr>F 0.0005). Figure 33 shows a two-dimensional canonical discriminant analysis of flies from four different counties in Oklahoma. The SAS discriminant analysis correctly identified 73% of the flies. In this analysis, the cuticular hydrocarbons of specimens from Lincoln County in northcentral Oklahoma, appeared to be more closely related to specimens from McCurtain County located in the southeastern corner of the state. Specimens collected from adjacent Wagoner and Muskogee Counties were not as similar as those collected from the other two counties. In this analysis, the specimens from adjacent counties are placed further apart from each other than specimens from distant counties. A possible reason for the variability might be these specimens were not collected at the same time. The temporal study presented earlier indicated a difference in the composition of the cuticular hydrocarbons from specimens collected at the same site over the season.

Fig. 33 Canonical discriminant analysis of cuticular hydrocarbons from *T. mularis* collected in malaise traps from four counties in Oklahoma.


5
4
3
2
1
0
-1
-2
-3
Can 1
-3
-2
-1
0
1
2
3
4
Can 2
+ Lincoln
■ Muskogee
▲ McCurtain
o Wagner

# CHAPTER VI

## SUMMARY AND CONCLUSIONS

Hydrocarbons comprised the major component of cuticular lipid in female *T. mularis*. The majority of cuticular hydrocarbons in *T. mularis* were saturated, with little or no unsaturated hydrocarbons and ranged from 27 to 40 carbons in length. These findings were consistent with those of Hoppe *et al.* (1990). The cuticular hydrocarbons and internal hydrocarbons of *T. mularis* were comprised of the same components, however, the relative amounts of the various molecules present differed in the two groups.

Preliminary studies revealed that cuticular hydrocarbon extracts analyzed by canonical discriminant analysis could be used to differentiate the closely related species *T. mularis* and *T. quinquevittatus*. However, these studies also indicated variability in the relative amounts of cuticular hydrocarbon molecules present in female *T. mularis* specimens which was attributed to differences in conditions of storage prior to hexane extraction.

In order to achieve the first objective of determining the effects of different collection and storage conditions on the amount and composition of extractable cuticular hydrocarbons from field collected *T. mularis*, two studies were designed. The results of these two studies further corroborated the hypothesis that the manner in which specimens were stored and handling conditions could affect the use of cuticular hydrocarbon analysis for identification of *T. mularis*. In the first study, cuticular hydrocarbon profiles of *T. mularis* collected from lake Carl Blackwell, Payne Co. OK, in 1977, 1978, and 1979 were found to be significantly different from profiles of *T. mularis* collected from the same location in 1995. Furthermore, cuticular hydrocarbon profiles of *T. mularis* from 1995 which were pinned 4 months were found to be significantly different from profiles of fresh specimens.

In the second study, cuticular hydrocarbon profiles of *T. mularis* collected and analyzed from 14 June 1995 which were fresh, were found to be significantly different from profiles of those which were pinned for a period of four months or freeze dried. These findings differed from those reported by Hoppe et al. (1990) who stated that analysis of extracts of fresh, frozen, and

pinned Tabanidae yielded nearly identical hydrocarbon profiles, although they did not use canonical discriminant analysis. The differences measured in the hydrocarbon extracts of *T. mularis* stored under different conditions were statistically significant. However, it should be noted the possibility exists that the canonical discriminant analysis used to analyze the data was too sensitive and selected for very small differences in the hydrocarbon profiles which may not correlate with any biological or functional differences. These two studies were the first to examine the effects of collection and storage conditions on the cuticular hydrocarbon profiles of an insect species.

The second objective, was designed to determine if seasonal distribution could play a role in the variability of cuticular hydrocarbon profiles of *T. mularis*. *Tabanus mularis* females were collected and extracted as fresh specimens throughout the summer of 1995 at weekly intervals. The canonical discriminant analysis revealed that there were significant differences in the composition of the cuticular hydrocarbon profiles of specimens collected on different dates. Again, this was the first study to reveal variability of cuticular hydrocarbons from a species collected throughout a season.

To accomplish the third objective, the cuticular hydrocarbon profiles of *T. mularis* from four different counties in Oklahoma were examined. Although there were significant differences among the profiles of *T. mularis* from the different counties, the results were not definitive. Hydrocarbon profiles of *T. mularis* from the most widely separated counties were deemed more closely related than *T. mularis* from neighboring counties. A possible explanation for this was that the *T. mularis* were collected from the four counties at different times during the summer of 1995. This factor alone could account for such results in the canonical discriminant analysis.

It is important to address the question of how much variability may be present in the cuticular hydrocarbons among members of a species before a conclusion regarding existence of strains, biotypes or subspecies may be reached. The larger question also occurs, regarding the extent of variability that can exist within specimens still considered to be members of the same species. This work indicates that considerable variation can occur in the results of cuticular hydrocarbon analysis of *T. mularis* with respect to storage conditions, as well as seasonal and

spatial collections which does not appear to be related to subspecies or biotypes of *T. mularis*. It merely suggests that there are significant and reproducible differences in the amounts of extractable cuticular hydrocarbons from specimens collected from different locations; specimens stored under different conditions; or specimens collected at different times of the year. There is too little known about the life history of this species, especially the larval habitat and food preferences that may attribute to differences in the cuticular hydrocarbon components, to draw any conclusions.

The main contribution of this work is to make researchers aware that questions of handling conditions, length of storage, and time of collection should be addressed when using cuticular hydrocarbon analysis for studying insect species. It should be noted that handling conditions prior to hexane extraction, length of storage and seasonal distribution contributes to variation in relative amounts of cuticular hydrocarbons profiles of *T. mularis* and may not affect this type of analysis on other insect species.

## References Cited


Bartelt, R.J., M.T. Armold, A.M. Schaner and L.L. Jackson. 1986. Comparative analysis of cuticular hydrocarbons in the *Drosophila virilis* species group. Comp. Biochem. Physiol. B. 83:731-742.

Bergman, D.K., J.W. Dillwith, R.K. Campbell and R.D. Eikenbary. 1990. Cuticular hydrocarbons of the Russian wheat aphid. Southwestern Entomologist. 15:91-100.

Blailock, T.T., G.J. Blomquist and L.L. Jackson. 1976. Biosynthesis of 2-methylalkanes in the crickets *Nemobius fasciatus* and *Gryllus pennsylvanicus*. Biochem. Biophys. Res. Commun. 68:841-849.

Bligh, E.G. and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Blomquist, G.J. and J.W. Dillwith. 1985. Cuticular lipids. *In* Comp. Insect Physiol. Biochem. Pharmacol. (Kerkut, G.A. and L.I. Gilbert, eds.) Pergamon Press (Oxford) 3:117-154.

Blomquist, G.J. and L.L. Jackson. 1973. Incorporation of labelled dietary n-alkanes into cuticular lipids of the grasshoppper *Melanoplus sanguinipes*. J. Insect Physiol., 19:1639-1647.

Blomquist, G.J., D.R. Nelson, and M. deRenobales. 1987. Chemistry, biochemistry and physiology of Insect cuticular lipids. Archives Insect Biochem. Physiol. 6:227-265.

Borror, D.J., C.A. Triplehorn and N.F. Johnson. 1989. An introduction to the study of insects. p. 549. Ft. Worth.

Brill, J.H. and W. Bertsch. 1985. An investigation of sampling methods for the analysis of insect cuticular hydrocarbons. J. Entomol. Sci. 20(4):435-443.

Burger, J.F. 1975. Horseflies of Arizona II. Notes on and keys to the adult Tabanidae of Arizona, genus Tabanus (Diptera). Proc. Ent. Soc. Wash., 77:15-33.

Carlson, D.A., S.K. Milstrey and S.K. Narang. 1993. Classification of tsetse flies *Glossina* spp. (Diptera: Glossinidae) by gas chromatographic analysis of cuticular components. Bull. of Entomol. Res. 83:507-515.

Carlson, D.A. and M.W. Service. 1979. Differentiation between species of the *Anopheles gambiae* Giles complex (Diptera: Culicidae) by analysis of cuticular hydrocarbons. Ann. Trop. Med. Parasitol. 73:589-592.

Carlson, D.A. and M.W. Service. 1980. Identification of mosquitoes of *Anopheles gambiae* species complex A and B by analysis of cuticular components. Science. 207:1089-1091.

Carlson, D.A. and J.F. Walsh. 1981. Identification of two west African black flies (Diptera: Simuliidae) of the *Simulium damnosum* species complex by analysis of cuticular paraffins. Acta Tropica. 38:235-239.

Carlson, D.A. and S.R. Yocom. 1986. Cuticular hydrocarbons from six species of Tephritid fruit flies. Archives of Insect Biochem. and Phys. 3:397-412.

Chen, C.S., M.S. Mulla, R.B. March and J.D. Chaney. 1990. Cuticular hydrocarbon patterns in *Culex quinquefasciatus* as influenced by age, sex, and geography. Bull. Soc. Vector Biol. 15:129-139.

Cooksey, L.M. 1985. Dispersion and population estimation of *Tabanus abactor* Philip (Diptera:Tabanidae) in northcentral Oklahoma. PhD thesis. Okla. St. University, Stillwater. 89p.

Dillwith, J.W., G.J. Blomquist and D.R. Nelson. 1981. Biosynthesis of the hydrocarbon components of the sex pheromone of the house fly, *Musca domestica* L. Insect Biochem. 11:247-253.

Dillwith, J.W., J.H. Nelson, J.G. Pomonis, D.R. Nelson and G.J. Blomquist. 1982. A $^{13}$C-NMR study of methyl-branched hydrocarbon biosynthesis in the house fly. J. biol. Chem. 257:11,305-11,314.

Downes, J.A. 1958. The feeding habits of biting flies and their significance in classification. Ann. Rev. Ent. 3:249-266.

Drees, B.M. 1993. The horse and deer flies (Diptera, Tabanidae) of Texas. Unpublished. Texas A&M Agric. Ext. Service.

Ewing, A.L., J.F. Stritzke and J.D. Kulbeth. 1984. Vegetation of the cross timbers experimental range, Payne county, Oklahoma. Oklahoma State University Agricultural Experiment Station Research Report p. 856.

Freeman, J.V. 1987. Immature stages of *Tabanus conterminus* and keys to the larvae and pupae of common Tabanidae from the United States east coast salt marshes. Ann. Ent. Soc. Amer., 80:613-623.

Freeman, J.V. and E.J. Hansens. 1972. Collecting larvae of the salt-marsh greenhead *Tabanus nigrovittatus* and related species in New Jersey: comparison of methods. Environ. Ent. 1:653-658.

Golden, K.A., L.J. Meinke and D.W. Stanley-Samuelson. 1992. Cuticular hydrocarbon discrimination of *Diabrotica* (Coleoptera: Chrysomelidae) sibling species. Ann. Entomol. Soc. Amer. 85(5):561-570.

Goodwin, J.T. 1973b. Immature stages of some eastern Nearctic Tabanidae (Diptera). III. The genus Tabanus Linnaeus. J. Georgia Ent. Soc. 8:82-99.

Goodwin, J.T. 1994. Immature stages of some eastern Nearctic Tabanidae (Diptera): Additional species of Tabanus Linnaeus. Southwestern Entomologist. 19:139-145.

Granett, P. and E.J. Hansens. 1957. Further observations on the effect of biting-fly control on milk production in cattle. J. Econ. Ent. 50:332-336.

Granger, C.A. 1970. Trap design and color as factors in trapping the salt marsh greenhead fly. J. Econ. Ent. 63:1670-1672.

Hansens, E.J., E.M. Bosler and J.W. Robinson. 1971. Use of traps for study and control of saltmarsh greenhead flies. J. Econ. Ent. 64:1481-1486.

Hine, J.S. 1907a. Second report upon the horse flies of Louisiana. Lousiana Agr. Exp. Sta. Bull. 93. 59p.

Hollander, A.L. and R.E. Wright. 1980a. Impact of Tabanids on cattle: blood meal size and preferred feeding sites. J. Econ. Ent. 73:431-433.

Hollander, A.L. and R.E. Wright. 1980b. Daily activity cycles of eight species of Oklahoma Tabanidae (Diptera). Envir. Entomol. 9:600-604.

Hoppe, K.L., J.W. Dillwith, R.E. Wright, and D.E. Szumlas. 1990. Identification of horse flies (Diptera: Tabanidae) by analysis of cuticular hydrocarbons. J. Med. Entomol. 27(4):480-486.

Howard, R.W. and G.J. Blomquist. 1982. Chemical ecology and biochemistry of insect hydrocarbons. Annu. Rev. Entomol. 27: 149.

Howell, D.E. and O. Schomberg. 1955. Oklahoma Tabanidae. J. Econ. Entomol. 48:763-764.

Jackson, L.L. and G.J. Blomquist. 1976. Insect waxes. In. Kolattukudy, P.E. (ed.) Chemistry and biochemistry of natural waxes. pp. 201-233. Amsterdam, Elsevier.

Jallon, J.M. and J.R. David. 1987. Variations in cuticular hydrocarbons among the eight species of the *Drosophila melanogaster* subgroup. Evolution. 41:294-302.

Kamhawi, S., R.P. Lane, M. Cameron, A. Phillips, P. Millgan, and D.H. Molyneux. 1992. The cuticular hydrocarbons of *Phlebotomus argentipes* (Diptera: Phlebotominae) from field populations in northern India and Sri Lanka, and their change with laboratory colonization. Bull. of Entomol. Res. 82:209-212.

Knox, P. and K.L Hays. 1972. Attraction of Tabanus spp. (Diptera: Tabanidae) to traps baited with carbon dioxide and other chemicals. Environ. Ent. 1:323-326.

Krinsky, W.L. 1976. Animal disease agents transmitted by horse flies and deer flies. J. Med. Ent. 13:225-275.

Kruger, E.L., C.D. Pappas and R.W. Howard. 1991. Cuticular hydrocarbon geographic variation among seven north American populations of *Aedes albopictus* (Diptera:Culicidae). J. Med. Entomol. 28(6):859-864.

Kruger, E.L. and C.D. Pappas. 1993. Geographic variation of cuticular hydrocarbons among fourteen populations of *Aedes albopictus* (Diptera:Culicidae). J. Med. Entomol. 30(3): 544-548.

Leucona, R., G. Riba, P. Cassier, and J.L. Clement. 1991. Alterations of insect epicuticular hydrocarbons during infection with *Beauveria bassiana* or *B. brongniartii*. J. Invertebr. Pathol. 58:10-18.

LeHane, M.J., 1991. Biology of blood-sucking insects. p.227. Harper Collins Academic. London

Leprince, D.J. and P. Jolicoeur. 1986. Response to carbon dioxide of *Tabanus quinquevittatus* Wiedemann females (Diptera: Tabanidae) in relation to relative abundance, parity, follicle development, and sperm and fructose presence. Can. Ent. 118:1273-1277.

Lockey, K.H. 1976. Cuticular hydrocarbons of *Locusta, Schistocera* and *Periplaneta* and their role in waterproofing. Insect Biochem. 6:457-472.

Lockey, K.H. 1978. The adult cuticular hydrocarbons of *Tenebrio molitor* L. and *Tenebrio obscurus* F. (Coleoptera: Tenebrionidae). Insect Biochem. 8:237-250.

Lockey, K.H. 1980. Insect cuticular hydrocarbons. Comp. Biochem. Physiol. 65B:457-462.

Lockey, K.H. 1988. Lipids of the insect cuticle, origin, composition and function. Comp. Biochem. Physiol. 89b. 595.

Major, M.A. and G.J. Blomquist. 1978. Biosynthesis of hydrocarbons in insects: decarboxylation of long chain fatty acids to n-alkanes in *Periplaneta.* Lipids. 13:323-328.

Milligan, P.J.M., A. Phillips, G. Broomfield and D.H. Molyneux. 1993. A study of the use of gas chromatography of cuticular hydrocarbons for identifying members of the *Anopheles gambiae* (Diptera: Culicidae) complex. Bull. of Entomol. Res. 83:613-624.

Miwa, T.K. 1963. Identification of peaks in gas-liquid chromatography. J. Amer. Oil Chem. Soc. 40:309-313.

Nelson, D.R., D.A. Carlson and C.L. Fatland. 1988. Cuticular hydrocarbons of tsetse flies II. *Glossina fuscipes fuscipes, G. palpalis palpalis, G.p. gambiensis, G. tachinoides* and *G. brevipalpis.* J. of Chem. Ecol. 14(3):963-987.

Nelson, D.R., J.W. Dillwith and G.J Blomquist. 1981. Cuticular hydrocarbons of the house fly *Musca domestica.* Insect Biochem. 11(2):187-197.

O'Connor, J.G, F.H. Burrow and M.S. Norris. 1962. Determination of normal paraffins in C20 to C32 paraffin waxes by molecular sieve adsorption. Anal. Chem. 34:82-85.

Pechuman, L.L. 1981. The horse flies and deer flies of New York (Diptera, Tabanidae). 2nd ed. Search: Agriculture. Cornell Universtiy Experiment Station. No. 18. Ithaca, N.Y.

Pechuman, L.L., D.W. Webb and H.J. Tesky. 1983. The Diptera, or true flies, of Illinois. I. Tabanidae. Ill. Nat. Hist. Survey 33(Art. 1). 122 pp.

Perich, M.J., R.E. Wright and K.S. Lusby. 1986. Impact of horse flies (Diptera: Tabanidae) on beef cattle. J. Econ. Entomol. 79:128-131.

Philip, C.B. 1965. Family Tabanidae. In A. Stone, C.W. Sabrosky, W.W. Wirth, R.H. Foote, and J.R. Coulson. A catalog of the Diptera of America north of Mexico. U.S. Dept. of Agric. Agric. Handb. No. 276.

Phillips, A., F. LePont, P. Desjeux, G.Broomfield and D.H. Molyneux. 1990. Separation of *Psychodopygus carrerai carrerai* and *P. yucumensis* (Diptera: Psychodidae) by gas chromatography of cuticular hydrocarbons. Acta Tropica. 47:145-149.

Phillips, A., P.J.M. Milligan, G. Broomfield and D.H. Molyneux. 1988. Identification of medically important Diptera by analysis of cuticular hydrocarbons. *In Biosystematics of Haematophagus Insects* (ed. M.W. Service), Systematics Association Special Volume No 37, pp. 39-59. Clarendon Press, Oxford. © The Systematics Association, 1988.

Phillips, A., A. Sabatini, P.J.M. Milligan, D. Boccolini, G. Broomfield and D.H. Molyneux. 1990. The *Anopheles maculipennis* complex (Diptera: Culicidae) comparison of the cuticular hydrocarbon profiles determined in adults of five Palaearctic species. Bull. Entomol. Res. 80:459-464.

Phillips, A., J.F. Walsh, R. Garms, D,H. Molyneux, P. Milligan and G. Ibrahim. 1985. Identification of adults of the *Simulium damnosum* complex using hydrocarbon analysis. Tropical Med. and Parasitol. 36:97-101.

Richards, L. and K.L. Knight. 1967. The horse flies and deer flies of Iowa (Diptera: Tabanidae). Iowa State J. Sci. 41(3):313-362.

Roberts, R.H. 1968. A feeding association between *Hippelates* (Diptera: Chloropidae) and Tabanidae on cattle; its possible role in transmission of anaplasmosis. Mosquito News 28:236-237.

Roberts, R.H. 1975. Relationship between the amount of carbon dioxide and the collection of Tabanidae in Malaise traps. Mosquito News. 35:150-154.

Roberts, R.H. 1976. The comparative efficiency of six trap types for the collection of Tabanidae (Diptera). Mosq. News. 36:530-535.

Rockel, E.G. and E.J. Hansens. 1970. Emergence and flight activity of salt-marsh horse flies and deer flies. Ann. Ent. Soc. Amer. 63:27-31.

Ryan, L., A. Phillips, P. Milligan, R. Lainson, D.H. Molyneux, and J.J. Shaw. 1986. Separation of female *Psychodopygus wellcomei* and *P. complexus* (Diptera: Psychodidae) by cuticular hydrocarbon analysis. Acta Tropica. 43:85-89.

SAS Institute Inc., SAS/STAT® Users Guide, Version 6, Fourth Edition, Volume 1, Cary, NC: SAS Institute Inc., 1989. 943pp.

Scholl, P.J. and J.J. Petersen. 1985. Biting flies. p. 58. In Williams, R.E. [ed.]. Livestock entomology. John Wiley & Sons, New York.

Steelman, C.D. 1976. Effects of external and internal arthropod parasites on domestic livestock production. Ann. Rev. Entomol. 21:155-178.

Thompson, P.H. 1969. Collecting methods for Tabanidae. Ann. Ent. Soc. Amer. 62:50-57.

Thompson, P.H. 1973a. Tabanidae (Diptera) of Texas I. Coastal marsh species, West Galveston Bay; incidence, frequency, abundance and seasonal distribution. Proc. Ent. Soc. Wash. 75(3):359-364.

Thompson, P.H. and P.C. Kranter. 1978. Rearing of Texas Tabanidae (Diptera). I. Collection, feeding and maintenance of coastal marsh species. Proc. Ent. Soc. Wash., 80:616-625.

Thompson, P.H., J.W. Holmes, Jr., P.C. Kranter, C.M. Ramey and M.J. Clary. 1979. Rearing of Texas Tabanidae (Diptera). II. Mass production of *Tabanus nigrovittatus* Macquart eggs and larvae. Southwestern Ent. 4:224-230.

Tidwell, M.C. 1973. The Tabanidae (Diptera) of Louisiana. Tulane Stud. Zool. and Bot. No. 18. Tulane Univ., New Orleans, La. 95 pp.

Toolson, E.C. and R. Kuper-Simbron. 1989. Laboratory evolution of epicuticular hydrocarbon composition and cuticular permeability in *Drosophila psuedoobscura*: Effects on sexual dimorphism and thermal-acclimation ability. Evolution. 43(2):468-473.

Vaz, A.H., G.J. Blomquist and R.C. Reitz. 1988. Characterization of the fatty acyl elongation reactions involved in hydrocarbon biosynthesis in the house fly, *Musca domestica* L. Insect Biochemistry. 18:177-184.

Wright, R.E., R. K. Whittle, and L.L. Pechuman. 1986. Range Extensions, New State Records and Annotated Checklist for Oklahoma Tabanidae (Diptera). J. Kansas Entomol. Soc. 59:235-245.

Wright, R.E., R.K. Whittle, M.J. Perich, and A.L. Hollander. 1984. Seasonal Occurrence of Horse Flies (Diptera: Tabanidae) in North Central Oklahoma. J. Kansas Entomol. Soc. 57:209 -215.

VITA

Robert A. Mihalovich

Candidate for the Degree of

Master of Science

Thesis: FACTORS AFFECTING CUTICULAR HYDROCARBON ANALYSIS FOR IDENTIFICATION OF FIELD POPULATIONS OF *TABANUS MULARIS* STONE (DIPTERA: TABANIDAE) IN OKLAHOMA

Major Field: Entomology

Biographical:

Personal Data: Born in Washington, Pennsylvania, On October 27, 1970, the son of Robert J. and Barbara Mihalovich.

Education: Graduated from Immaculate Conception High School, Washington, Pennsylvania in June 1989; received Bachelor of Arts degree in Biology from Washington and Jefferson College, Washington, Pennsylvania in May 1993. Completed the requirements for the Master of Science degree with a major in Entomology at Oklahoma State University in May 1996.

Experience: Raised in a suburb of Pittsburgh, Pennsylvania; employed as a farm laborer during summers; employed by Washington Steel Corp. and the Department of Biology at Washington and Jefferson College as an undergraduate; employed as a graduate teaching assistant; Oklahoma State University, Department of Entomology, 1993 to present.

Professional Memberships: Entomological Society of America, Phi Alpha Theta History Honor Society.