(Z)-11-Octadecenyl acetate in Drosophila funebris : formation, transfer, catabolism and aggregation activity
by Russell Dean Leu

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry
Montana State University

Abstract:
(Z)-11-Octadecenyl acetate (Z11 - 18 : Ac) was identified as the most abundant (2300 ng/fly) hexane extractable component of the ejaculatory bulb of sexually mature virgin male D. funebris. Virgin female flies did not have Z11 - 18 : Ac at any age. There was a rapid increase in Z11 - 18 : Ac in virgin male flies during the first three days after eclosion. In mature male flies, about, 1500 ng of Z11 - 18 : Ac was in the ejaculatory bulb and about 800 ng was on the surface of the fly. During mating about 1200 ng of Z11 -18 : Ac was transferred to the female fly, but was not transferred into the reproductive tract. The female fly then looses approximately 60 ng of the Z1 1-18:Ac to the media. This amount is independent of the time spent in the holding vial. The amount of Z11 - 18:Ac on mated females decreased to undetectable levels at 12 hours post-mating. Concurrent with the Z11-18:Ac decrease there is an increase in the concentration of 14, 16 and 18 carbon fatty acids. The female is able to metabolize external Iy applied Z11 -18:Ac, vaccenol , vaccenic acid, oleyl acetate and stearyl acetate. Sexually immature (6-8 hours old) virgin females can metabolize applied Z11-18:Ac. A combination of Z11 -18:Ac and volatile, polar components from the male hexane extract comprise the aggregation pheromone in Eu. funebris.

(Z)-11-OCTADECENYL ACETATE IN *DROSOPHILA FUNEBRIS*: FORMATION, TRANSFER, CATABOLISM AND AGGREGATION ACTIVITY

by

Russell Dean Leu

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biochemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

March 1988

APPROVAL

of a thesis submitted by

Russell Dean Leu

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate studies.

February 19, 1988
Date

Leroy L Jacobson
Chairperson, Graduate Committee

Approved for the Chemistry Department

Feb. 26, 1988
Date

Bradford Philip Mundy
Head, Chemistry Department

Approved for the College of Graduate Studies

3-7-88
Date

Michael P. Malone
Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Dean of Libraries when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Russell Lee

Date February 19, 1988

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

(Z)-11-Octadecenyl acetate (Z11-18:Ac) was identified as the most abundant (2300 ng/fly) hexane extractable component of the ejaculatory bulb of sexually mature virgin male D. funebris. Virgin female flies did not have Z11-18:Ac at any age. There was a rapid increase in Z11-18:Ac in virgin male flies during the first three days after eclosion. In mature male flies, about 1500 ng of Z11-18:Ac was in the ejaculatory bulb and about 800 ng was on the surface of the fly. During mating about 1200 ng of Z11-18:Ac was transferred to the female fly, but was not transferred into the reproductive tract. The female fly then looses approximately 60 ng of the Z11-18:Ac to the media. This amount is independent of the time spent in the holding vial. The amount of Z11-18:Ac on mated females decreased to undetectable levels at 12 hours post-mating. Concurrent with the Z11-18:Ac decrease there is an increase in the concentration of 14, 16 and 18 carbon fatty acids. The female is able to metabolize externally applied Z11-18:Ac, vaccenol, vaccenic acid, oleyl acetate and stearyl acetate. Sexually immature (6-8 hours old) virgin females can metabolize applied Z11-18:Ac. A combination of Z11-18:Ac and volatile, polar components from the male hexane extract comprise the aggregation pheromone in D. funebris.

INTRODUCTION

D. funebris is one of the most cold adapted cosmopolitan species (1),(2) of *Drosophila* found primarily in temperate woodlands (1) and is common at the most southern and northern latitudes (3). Although it is considered a facultative fungal species (4), *D. funebris* does well on a synthetic Drosophila diet (Instant Drosophila Medium 4-24, Carolina Biological). Many closely related species are obligate fungal feeders (4) but *D. funebris* will not hybridize with them (2).

Previous studies of *D. funebris* have included dispersal rates (5),(6), (7); fitness parameters (8), (9), (1) and accessory gland secretory proteins (10), (11).

Aggregation using aggregation pheromones is probably a general phenomenon in the *Drosophila*. Male-produced aggregation pheromones have been demonstrated in seven species of the *D. virilis* species group (12), (13), (14), (15), in *D. melanogaster* (16), in *D. simulans* (17), in *D. malerkotliana* (18), in *D. ananassae* and *D. bipectinate* (19), in *D. mauritiana, D. yakuba* and *D. rajasekari* (20), in *D. hydei* (21), and in *D. mulleri* (22), as shown in Table 1.

Table 1. Drosophila species with identified aggregation pheromones.

GENUS DROSOPHILA

SUBGENUS SOPHOPHORA

GROUP MELANOGASTER

SUBGROUP MELANOGASTER	SUBGROUP SUZUKII	SUBGROUP ANANASSAE
-melanogaster	-rajasekai	-ananassae
-mauritiana		-bipectinata
-simulans		-malerkotliana
-yakuba		

SUBGENUS DROSOPHILA

GROUP VIRILIS	GROUP REPLETA		GROUP FUNEBRIS
	SUBGROUP MULLERI	SUBGROUP HYDEI	
-americana	-mulleri	-hydei	-funebris
-borealis			
-littoralis			
-lummei			
-novamexicana			
-texana			
-virilis			

D. melanogaster, *D. simulans*, *D. mauritiana*, *D. yakuba*, *D. rajasekari*, *D. ananassae*, *D. bipectinata*, and *D. malerkotliana* are all in the *melanogaster* group of the subgenus Sophophora and all of these species use Z-11-octadecenyl acetate (Z11-18:Ac) as their aggregation pheromone, except *D. malerkotliana* and *D. bipectinata* that use Z11-20:Ac. The rest of the species studied to date are in the Drosophila subgenus and the aggregation pheromones are hydrocarbons, esters, and ketones, and usually a blend of two classes of these compounds comprises the aggregation pheromone. In all cases the pheromone attracts nearly equal

numbers of both sexes in the wind-tunnel olfactometer, and the pheromone is synergistic with food related odors. In most species, the aggregation pheromone is present in the mature male ejaculatory bulb, transferred to the female reproductive tract during mating, and transferred by the females to the food media within hours after mating (16), (17), (18), (19), (20).

Non-pheromonal studies of Z11-18:Ac include work on D. melanogaster where it was reported to inhibit courtship and mating (23), but this was proven to be incorrect (24), (25). Z11-18:Ac has been quantitated in virgin male and mated female D. melanogaster (16), (24), (25). Males transfer approximately 300 ng (20% of their Z11-18:Ac) into the female's reproductive tract with approximately 20 ng located on the cuticle. The female then loses a majority of the transferred Z11-18:Ac to the vial within 6 hours.

No previous pheromonal work with D. funebris has been reported. D. funebris in the Sophophora subgenus is a distant relative of the melanogaster group species studied thus far. Experiments with D. funebris are patterned after previous pheromone studies (16), (17).

Objectives

The most abundant hexane extractable compound of the ejaculatory bulb will be characterized including: 1) identification, 2) rate of synthesis, 3) transfer to the female, 4) loss by the female, and 5) involvement in the aggregation pheromone system.

METHODS AND MATERIALS

D. funebris, wild type, from the (Department of Biology-University of Milan, Milan, Italy) were raised on a diet of yeasted Instant Drosophila medium 4-24 (Carolina Biological Supply Co., Burlington, North Carolina) at ambient lab temperatures using a 16 hour light and 8 hour dark cycle. At less than 24 hours after eclosion, flies (anesthesized with carbon dioxide) were separated by sex. Approximately 10 flies were put into a rearing vial (10 cm x 3 cm ID) until a specified age. *D. melanogaster* Canton S were reared and handled as reported previously (16).

Ejaculatory Bulb Removal and Analysis

Males were killed by placing them at -10° C. for approximately 30 minutes. A thin dissecting pin was used to fasten the fly to a cork board. Under 20X magnification, the tip of the abdomen (near the genitalia) was grasped between two pins and this section of tissue which included the ejaculatory bulb was removed. The ejaculatory bulb was carefully separated from the surrounding tissue and placed in a 0.5 ml conical vial containing 10 ul of hexane and 1 ug of nonadecane as a quantitative internal standard. A pin

point was used to smash the bulb, and release the contents for extraction.

The hexane extract (2-3 ul) was analyzed in a Varian 3700 gas chromatograph fitted with a 15 meter Megabore DB-1 capillary column (J and W Scientific, Folsom, CA) and flame ionization detector. The temperature program was initial temperature 130°C, increased at 10°C/min to a final temperature of 300°C. Chromatographic retention times of synthetic standards of the hydrocarbons, esters and ketones previously observed as aggregation pheromones were compared to the retention time of the major chromatographic peak in the D. funebris ejaculatory bulb extract. Compounds with similar retention times to the peak from D. funebris were chromatographed on a 30 meter DB-225 capillary column (J and W Scientific, Folsom, CA) programmed from 130 to 200°C at 5°/min.

Electron impact mass spectra were obtained on a VG MM16 mass spectrometer using a 30 meter DB-5 capillary GC column for introduction of the sample. The double bond was located by ozonolysis and GC of the products (26).

Extraction of Whole Flies

The ejaculatory bulb compound could be extracted from whole mature male flies by soaking them for 45 minutes in 10 ul of hexane containing 1 ug of nonadecane as a quantitative internal standard. Likewise, the remainder of the fly after

removal of the ejaculatory bulb was soaked in hexane for 45 minutes and the extract analyzed by GC.

Formation of the Ejaculatory Bulb Compound with Age

Within two hours of eclosion and each day thereafter up to 6 days, the ejaculatory bulbs of males were removed, extracted and analyzed. The remainder of the flies was also extracted and analyze.

Transfer During Mating

Seven day-old flies were used in the mating experiments. Flies to be mated were placed in (9mm diameter X 6 mm height) chambers without the aid of anesthesia. Immediately upon completion of mating or at various predetermined times after copulation began, the individual flies were killed by heavy ether anesthesia, then placed into separate 0.5-1.0 ml conical vials and stored at -10°C until subsequent extraction and GC analysis.

Female Reproductive Tract Removal

The reproductive tract of mated female flies was removed by first fastening a fly to a cork board with a pin. The ovipositor was clasped with a pair of forceps and pulled out. Two dissecting pins were used to separate the reproductive tract excluding the ovaries from the intestine. The reproductive tract excluding the ovaries was placed into

a 1 ml conical vial with 10 ul of hexane containing 1 ug of nonadecane as a quantitative internal standard. Analysis by GC was performed as previously described.

Ejaculatory Bulb Compound Loss by the Female

Immediately after completion of mating, or at a specified time, females were removed from the mating chamber using carbon dioxide anesthesia. Ten females were placed in a 4 ml conical vial fitted with a wire mesh cap. After a specified time, the vial containing the females was placed in a freezer at -10°C. for 30 minutes, the females were removed and placed into a separate conical vial for extraction and GC analysis as previously described. The vial was washed 3 times with 100 ul of hexane. After concentrating the extract under nitrogen, 1 ug of nonadecane, a quantitative internal standard was added. GC-analysis was performed as previously described.

Application of Compounds to Virgin Females

Seven day old virgin females were refrigerated for approximately 10 minutes to make them easier to handle. They were then placed in a petri dish which sat on crushed ice. Using a 1.0 ul syringe (Hamilton Co.) 0.2-0.3 ul of acetone containing a specified amount of material was applied to the posterior end of the fly. Ten flies were put into a 4 ml conical vial fitted with a wire mesh cap for a

predetermined period of time. Flies and vial were extracted as previously described, and analyzed in a Varian 3700 gas chromatograph fitted with a 30 meter Megabore DB-225 capillary column (J&W Scientific, Folsom, CA) and flame ionization detector. The temperature program was initial temperature, 150° increased at 2° C/min to a final temperature of 200°. An equal volume of Meth-Prep I (Applied Science) to methylate the fatty acids was coinjected with the sample.

Collection of Volatiles from Hexane Extracts

A 2 cm long column of Tenax porous polymer 35/60 Mesh (Applied Science) was formed in a capillary tube (2mm ID X 95mm L) with glass wool placed on either side. An apparatus was assemblied so that a gentle stream of nitrogen at ambient temperature evaporated a 0.5 ml sample, causing the vapors to pass into the Tenax column. After complete evaporation which took approximately 10 minutes, the Tenax was eluted with 250 ul of pentane and the resulting extract was quantitated by GC and used in further tests.

Bioassays

Flies for the bioassays were removed from the rearing jars when 0-2 days old, starved overnight in the wind-tunnel olfactometer and tested the following morning. The bioassay procedure and apparatus were fully described by Bartelt and

Jackson (12). Briefly, the wind-tunnel olfactometer was large enough to allow free flight and was stocked with approximately 1000 flies. A sample to be tested was applied to a filter paper strip inserted around the lip of a glass vial. Two vials to be compared were placed on the floor of the olfactometer in the upwind end. Each vial contained a drop of water, which was not itself attractive but which caused the flies that entered the vial to remain throughout the test. Tests lasted for 3 minutes after which the vials were capped and flies counted. Each bioassay experiment used the balanced incomplete block design, in which the treatments were tested in pairs in all possible combinations. The bioassay data was transformed to the log(x+1) scale before analysis to stabilize variance and analysis was done by the method of Yates (27). Normally, 12 tests could be run before the number of flies became too low to give good results.

RESULTS AND DISCUSSION

Identification

The hexane extract of mature male ejaculatory bulbs contained only one GC peak of appreciable size. Comparison of retention times on the non polar DB-1 column of previously used synthetic compounds showed a match with (Z)-11-octadecenyl acetate (Z11-18:Ac). The ejaculatory bulb compound and Z11-18:Ac also had matching retention times on the polar DB-225 column. Mass-spectra and GC analysis of the ozonolysis products of the ejaculatory bulb compound and Z11-18:Ac were identical. All evidence supported the structure of the ejaculatory bulb compound as (Z)-11-octadecenyl acetate.

Z11-18:Ac has been identified from the ejaculatory bulb of a number of species in the melanogaster group, but this was the first report of Z11-18:Ac outside the melanogaster group of the Sophophora subgenus.

Formation

There was no detectable Z11-18:Ac present in virgin females of any age; however, there was a dramatic increase in Z11-18:Ac in male flies during the first three days, as shown in Figure 1. After three days, the level of

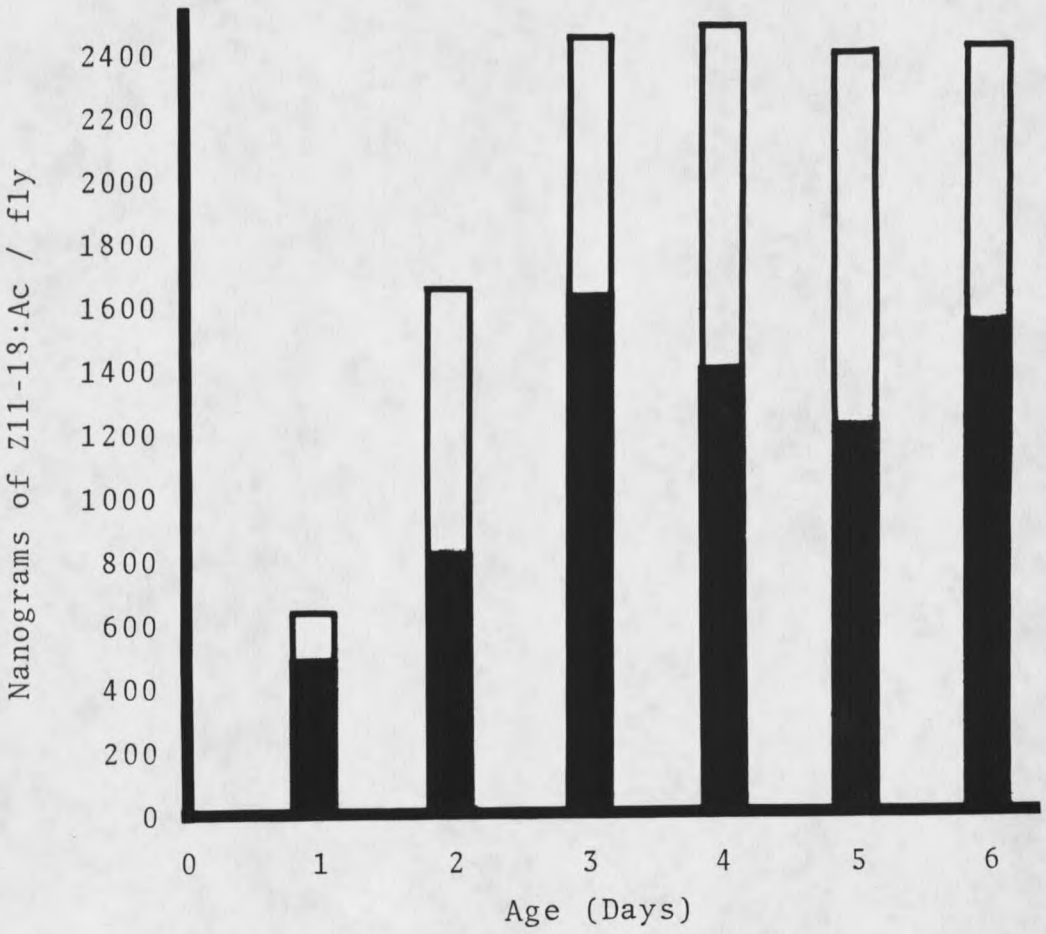

Figure 1. (Z)-11-Octadecenyl acetate (Z11-18:Ac) in male D. funebris with age. Shaded areas represent Z11-18:Ac in the ejaculatory bulb while clear areas represent Z11-18:Ac on the rest of the fly (N=2 sets of 3 flies).

Z11-18:Ac reached a plateau level of approximately 2300 ng/fly. In comparison, ejaculatory bulbs of *D. melanogaster* had approximately 1600 ng/fly of Z11-18:Ac (24), hexane extracts of whole flies of *D. melanogaster* had 1400 ng/fly at age 5 days (16) and *D. simulans* had 1000 ng/fly (17). In male *D. funebris* of all ages, most of the Z11-18:Ac was located in the ejaculatory bulb, however after 2 days of age there was approximately 800 ng/fly of Z11-18:Ac on the remainder of the fly, as shown in Figure 1. This was confirmed by making two 1 second hexane dips of mature male flies which removed 820 ng of Z11-18:Ac.

Transfer During Mating

Z11-18:Ac (1000-1400 ng) was transferred from virgin male *D. funebris* to the female during mating. Over half of the Z11-18:Ac was transferred within the first five minutes of mating, as illustrated in Figure 2, with a maximum of it being transferred in the first 13 minutes of an approximately 16 minute mating. *D. funebris* produced some unexpected results. The female reproductive tracts contained 240 $\pm$ 45 ng Z11-18:Ac/fly which was approximately 20% of the transferred Z11-18:Ac. After three minutes, the amount present in the reproductive tract was independent of time. Eighty percent of the Z11-18:Ac transferred to *D. funebris* females was easily extractable with hexane, suggesting that it was on or near the surface of the fly, as

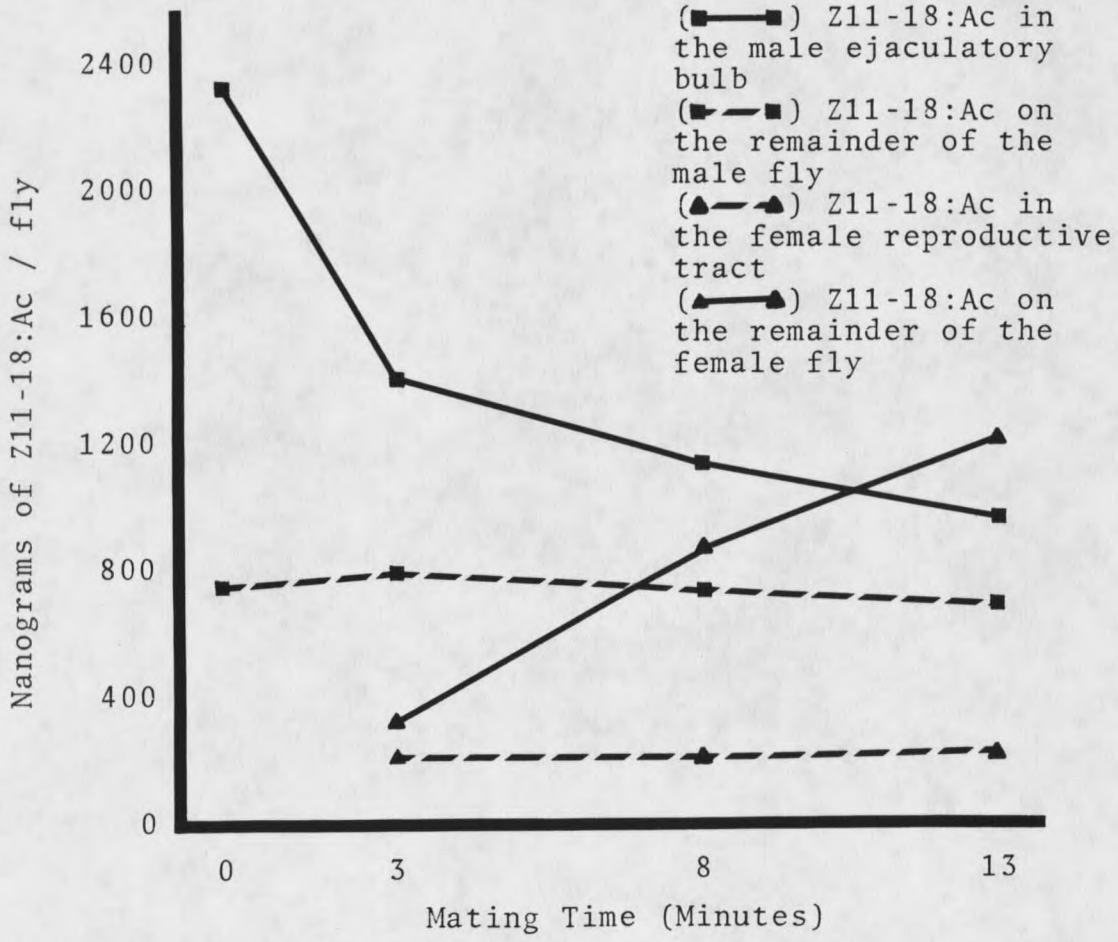

Figure 2. Seven day old D. funebris matings with the subsequent removal of the ejaculatory bulb, reproductive tract and hexane soak of the fly. (N=3 sets of 3 flies)

shown in Table 2 and Figure 2, unlike what was observed with D. melanogaster. Transfer of Z11-18:Ac in D. funebris must be occurring differently than D. melanogaster. It appeared that the female reproductive tract became "filled" within 3 minutes and the rest of the transferred Z11-18:Ac was deposited on or near the surface of the cuticle. Since the mated pair was still coupled when they were frozen, there should be no way for the female to lose the Z11-18:Ac from her reproductive tract.

Table 2. A comparison of the amounts of Z11-18:Ac sequentially extracted from D. funebris and D. melanogaster mated females.

	Z11-18:Ac (ng/fly) (N=7 sets of 3 flies)			
Mated female flies	1st one sec dip	2nd one sec dip	45 min soak	Total
D. funebris	560±210	260±80	170±90	980±320
D. melanogaster	42±9	15±5	360±80	410±80

In D. melanogaster, approximately 300 ng of Z11-18:Ac was transferred (24) and the transfer was complete within the first six minutes of an approximately 20 min mating (25). In D. melanogaster the Z11-18:Ac was deposited into the female's reproductive tract during mating with only a small amount (60 ng) found on the female's cuticle (Table 2; 25). In comparison, two 1 second hexane dips of mated D.

funebris females removed approximately 800 ng of Z11-18:Ac but only approximately 60 ng of Z11-18:Ac from *D. melanogaster* mated females, as presented in Table 2.

The possibility was considered that mated *D. funebris* females were releasing the Z11-18:Ac to their legs for transfer to their food source. Removal and extraction of legs from mated females revealed the legs had 81±68 ng/fly while the remainder of the fly had 990 ± 230 ng/fly of Z11-18:Ac. This small amount could result from rubbing her legs against her cuticle.

If *D. funebris* males were only depositing a small portion of the Z11-18:Ac into the female's reproductive tract, how was such a large quantity transferred to the female? One method could be cuticle to cuticle. Interrupting a mating just as the pair started (0-1 minute) showed that there was no additional Z11-18:Ac on the surface of mating male flies than on virgin flies, as shown in Table 3. The males had transferred over 200 ng of Z11-18:Ac to the females with no corresponding increase in the level of Z11-18:Ac extracted from their cuticle. The males were not transferring a large quantity of Z11-18:Ac to their cuticle and then "rubbing it" on the female's cuticle. Neither was the female using her legs to "rub" the Z11-18:Ac from the males's cuticle onto her cuticle. The small amount (80 ng) of Z11-18:Ac located on her legs could result from a

preening action with the Z11-18:Ac being removed from her own cuticle.

Table 3. Recovery of Z11-18:Ac from mated and virgin *D. funebris*.

Treatment	ng Z11-18:Ac/fly (± SD) (N=5 sets of 3 flies)		
	0-1 min mating	Complete mating	Virgin fly
1st one sec dip of male	120±50	150±40	120±120
2nd one sec dip of male	220±120	140±50	180±110
45 min soak of male	740±430	450±240	750±260
45 min soak of female	240±90	1260±320	

Catabolism

Within 24 hours, the female had lost all of the transferred Z11-18:Ac with approximately 75% lost within 3 hours, as illustrated in Figure 3. Extraction of the female holding vial showed that <100 ng of Z11-18:Ac was deposited in the vial. The transfer to the vial was independent of time and showed a slight increase for the first 9 hours and then decreased. This amount could be rubbed from the cuticle as the female came into contact with the surface of the vial. Females were "losing" Z11-18:Ac, but not to their surroundings. In comparison, when the male deposits

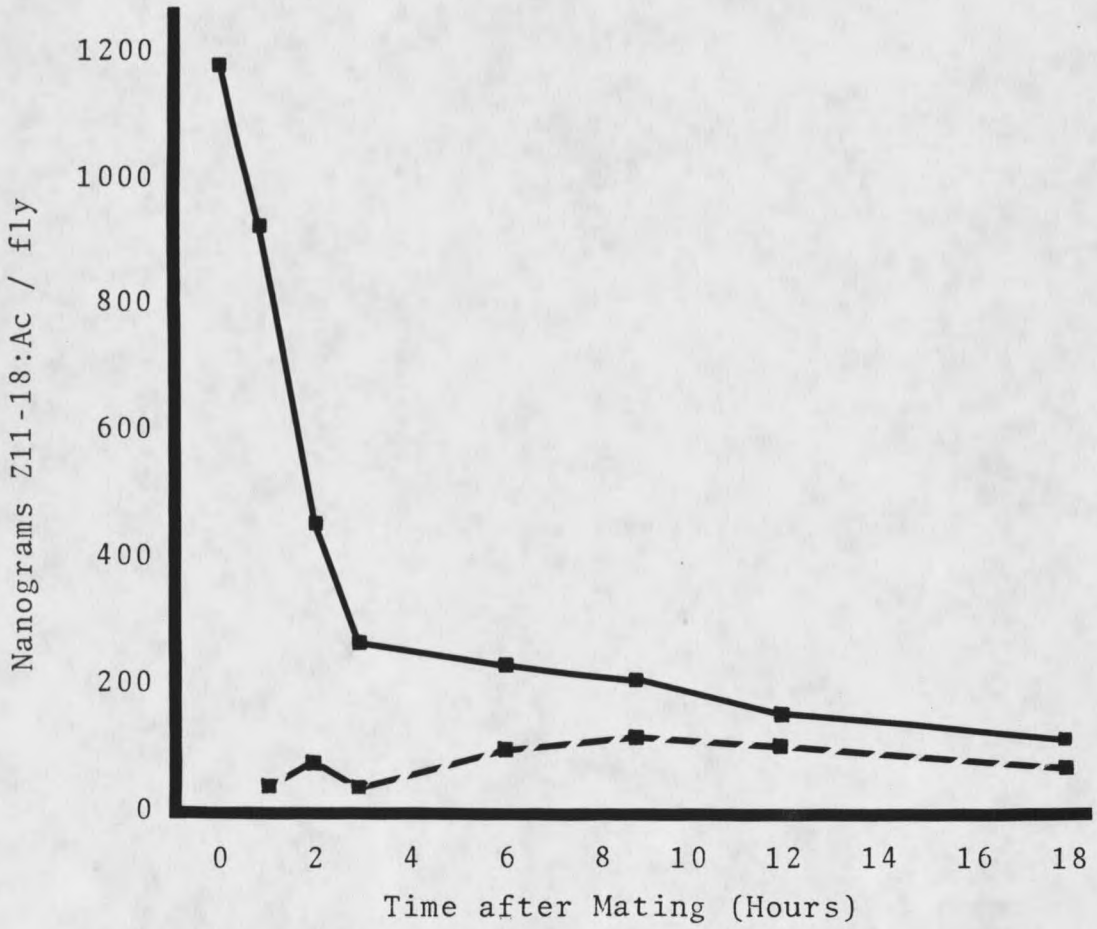

Figure 3. Loss of (Z)-11-Octadecenyl acetate (Z11-18:Ac) from mated D. funebris females with time. (■——■) represents Z11-18:Ac on the female. (■– –■) represents Z11-18:Ac emitted by the female into the vial. (N=1 set of 10 flies)

the Z11-18:Ac into the female's reproductive tract, within 6 hours after mating both D. simulans (17) and D. melanogaster (16) lose a majority of the transferred Z11-18:Ac to their holding vials.

Application of Z11-18:Ac

Females may be sequestering the Z11-18:Ac internally or metabolizing it. A dose of 1100 ng of Z11-18:Ac in acetone was applied to 7 day old virgin females and the surface extracts of the flies were analyzed hourly. Compared with mated females, a similar rate of Z11-18:Ac loss by virgin females occurred, as shown in Figure 4, with a rapid disappearance for the first four hours and then a gradual loss. The quantity of Z11-18:Ac in the holding vials was similar to the quantity emitted by mated females. Approximately 5% of the applied Z11-18:Ac was lost to the vial and the amount deposited was independent of time. Virgin females treated the applied Z11-18:Ac similarly to the transferred Z11-18:Ac of mated females, but it is not clear whether the Z11-18:Ac was sequestered internally or metabolized.

Flies to which Z11-18:Ac had been applied showed an increased level of 14, 16 and 18 carbon fatty acids reaching a peak of approximately 600 ng at 3 hours followed by a rapid decrease from 3 to 4 hours and then a slow decrease to normal levels within 12 hours, as shown in Figure 4.

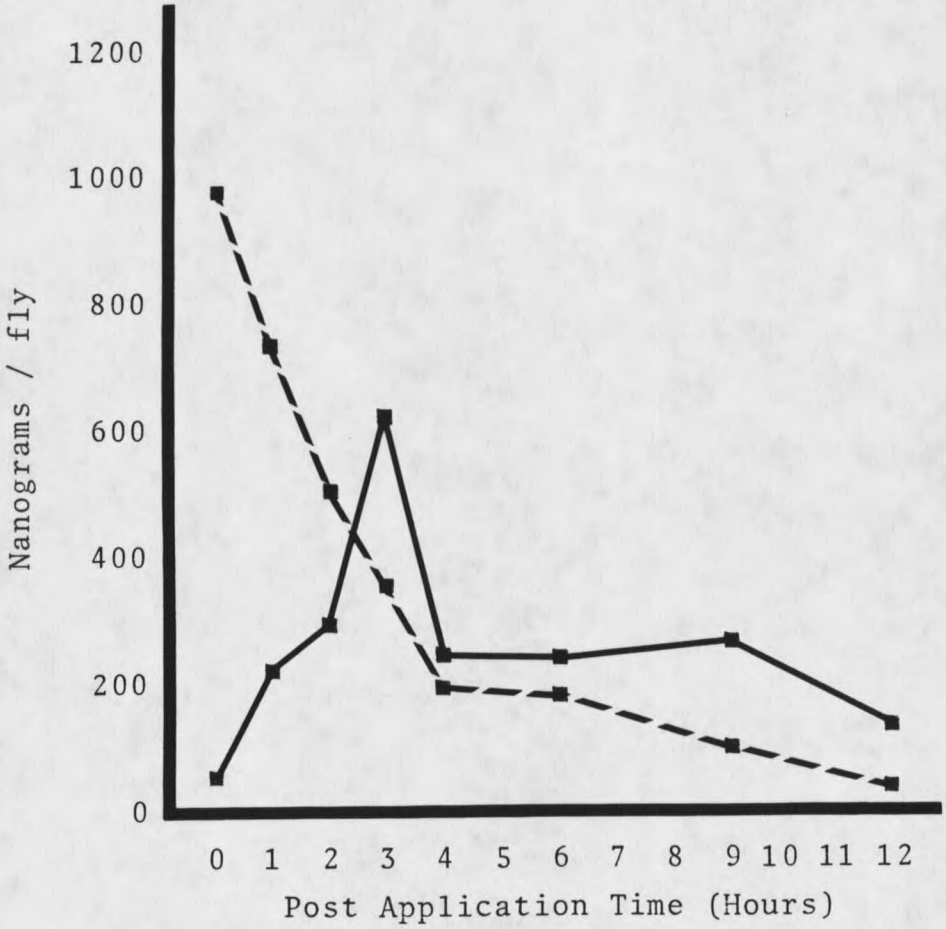

Figure 4. Application of 1100 ng of (Z)-11-Octadecenyl acetate (Z11-18:Ac) to 7 day old virgin female D. funebris. (■—■) represents Z11-18:Ac remaining on the fly. (■——■) represents the total amount of cuticular fatty acids. (N=5 sets of 10 flies)

Female flies to which only acetone was applied showed <100 ng of 14, 16, 18 carbon fatty acids whether the female was extracted and analyzed immediately (0 hour) or allowed to remain in the holding vial for 3 hours. Soaking time was critical in determining the concentrations of these fatty acids. A 45 minute hexane soak removed the external compounds without leaching out very much of the internal compounds. It was important to remove only surface compounds since these fatty acids were also present internally in both the male and female flies and significant amounts could be extracted with a 3 hour or longer hexane soak.

Since the concentration of these fatty acids increased and then decreased rather than remain relatively constant, further metabolic processes were indicated. Using a much lower starting temperature for the GC analysis, no new peaks appeared as the concentration of the three fatty acids decreased. This implied that the three fatty acids were either degraded very rapidly or else the female was transferring them internally and the 45 minute hexane soak was not removing them. A 24 hour hexane soak removed significantly more of the three fatty acid products but it was not known whether the products were naturally there or whether some of the applied material was taken internally.

If 1100 ng of Z11-18:Ac was applied to very young (6-8 hour old) virgin females, they possessed the same amount

of Z11-18:Ac (approximately 350 ng) after 3 hours and lost the same amount to the vial (approximately 20 ng) as the mature flies did. Thus, the transfer system was functional at a very young age.

Further tests to show that Z11-18:Ac was metabolized by the female involved the application of synthetic vaccenic acid and vaccenol to the female. A degradation process of Z11-18:Ac would presumably involve hydrolyzing the acetate ester, oxidizing the alcohol to an acid, and then oxidizing the acid to shorter chain compounds. If vaccenic acid and vaccenol were on the degradation pathway then the enzyme system(s) involved would treat them similar to Z11-18:Ac and metabolize them.

Application of Vaccenic Acid

When a large dosage (1000 ng was utilized too rapidly to accurately measure) of vaccenic acid (2500 ng) was applied to a mature virgin female, it was utilized very rapidly, as illustrated in Figure 5. Within 2 hours approximately 80% of the applied vaccenic acid was "lost" from the cuticle A 24 hour hexane soak showed approximately 1100 ng of vaccenic acid was present within the fly. The ratio of internal vaccenic acid to oleic acid was relatively constant for the first 2 hours after application then decreased rapidly with no vaccenic acid detected after 5 hours. Hexane rinses of the holding vials showed that no vaccenic

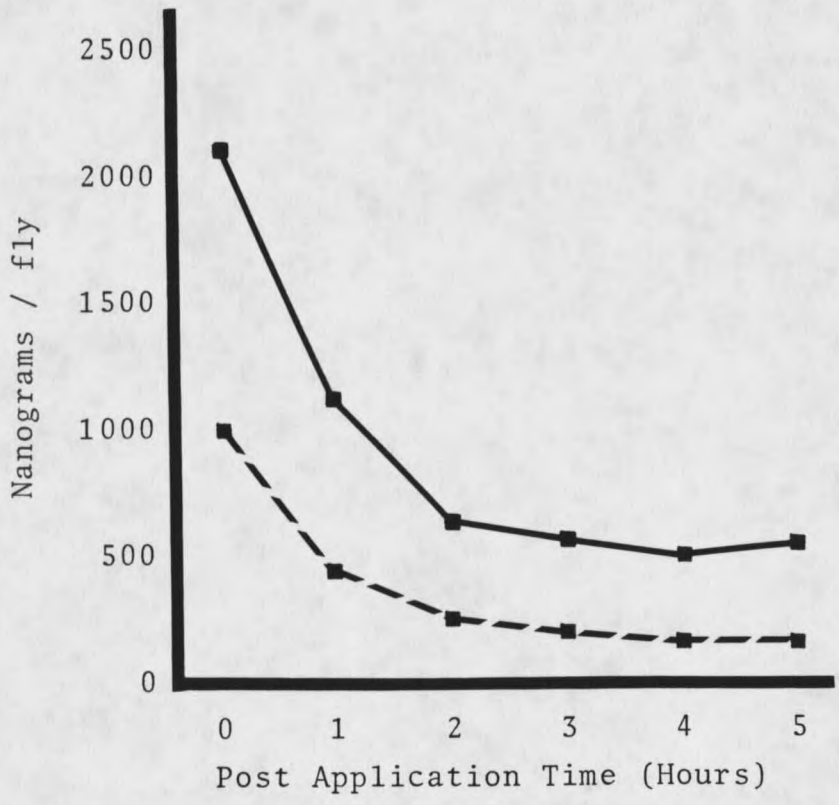

Figure 5. Application of 2500 ng of vaccenic acid (■——■) or 1000 ng of vaccenol (■– –■) to 7 day old D. funebris females. (N= 1 set of 10 flies)

acid was lost to the surroundings. In addition, no Z11-18:Ac or vaccenol was seen in any of the chromatograms.

Comparing the free fatty acid levels extracted by the 24 hour hexane soak after vaccenic acid was applied, showed that the 16:0 and 16:1 fatty acid levels were relatively constant with time (approximately 1.3 times the 18:1 levels) while the 14:0 and 14:1 levels increased slightly with time. Thus, the decrease in vaccenic acid levels in the fly does not change the internal fatty acid profile very much.

Application of Vaccenol

When 1000 ng of vaccenol was applied to a mature female, the disappearance of vaccenol was similar to the disappearance of vaccenic acid with a rapid loss within the first 3-4 hours and then a gradual loss, as shown in Figure 5. In addition, the 45 minute hexane soak consistently removed a small amount of Z11-18:Ac (approximately 30 ng) and vaccenic acid (approximately 10 ng). The enzyme system(s) involved in this transformation seems to be able to both oxidize the alcohol to the acid and acetylate the alcohol to the acetate ester. While the 45 minute hexane soak removed a small amount of Z11-18:Ac from the surface, a 24 hour hexane soak removed no internal Z11-18:Ac or vaccenic acid. Rinses of the vials showed that little if any vaccenol was released tothe surroundings. Neither vaccenicacid nor Z11-18:Ac was detected invial rinses.

Application of similar acetates

Since the female was able to metabolize Z11-18:Ac and its degradation products, the degradation of two closely related acetates (stearyl acetate, an 18 carbon saturated acetate and oleyl acetate, an 18 carbon unsaturated acetate with the double bond located in the 9 position) were tested. Utilization of the two acetates applied at 1000 ng/fly was similar to the previously applied compounds with a rapid initial loss that tapers off, as shown in Figure 6. A small amount of both compounds (approximately 50 ng) was deposited into the holding vial. This amount was relatively constant and did not increase with time. Oleyl acetate was metabolized slightly slower than Z11-18:Ac or stearyl acetate. Thus, a non-specific enzyme system(s) was involved, because lack of a double bond or movement of the double bond doesn't affect the enzyme's ability to metabolize the applied compounds.

Application of Z11-18:Ac to D. melanogaster

The ability of D. funebris to metabolize Z11-18:Ac and closely related compounds lead us to investigate D. melanogaster. Z11-18:Ac was also the aggregation pheromone in this species (16). When 500 ng of Z11-18:Ac was applied to virgin females, the same utilization pattern as in D. funebris occurred, with 90% of the Z11-18:Ac lost from the cuticle within 5 hours, as shown in Figure 7. In

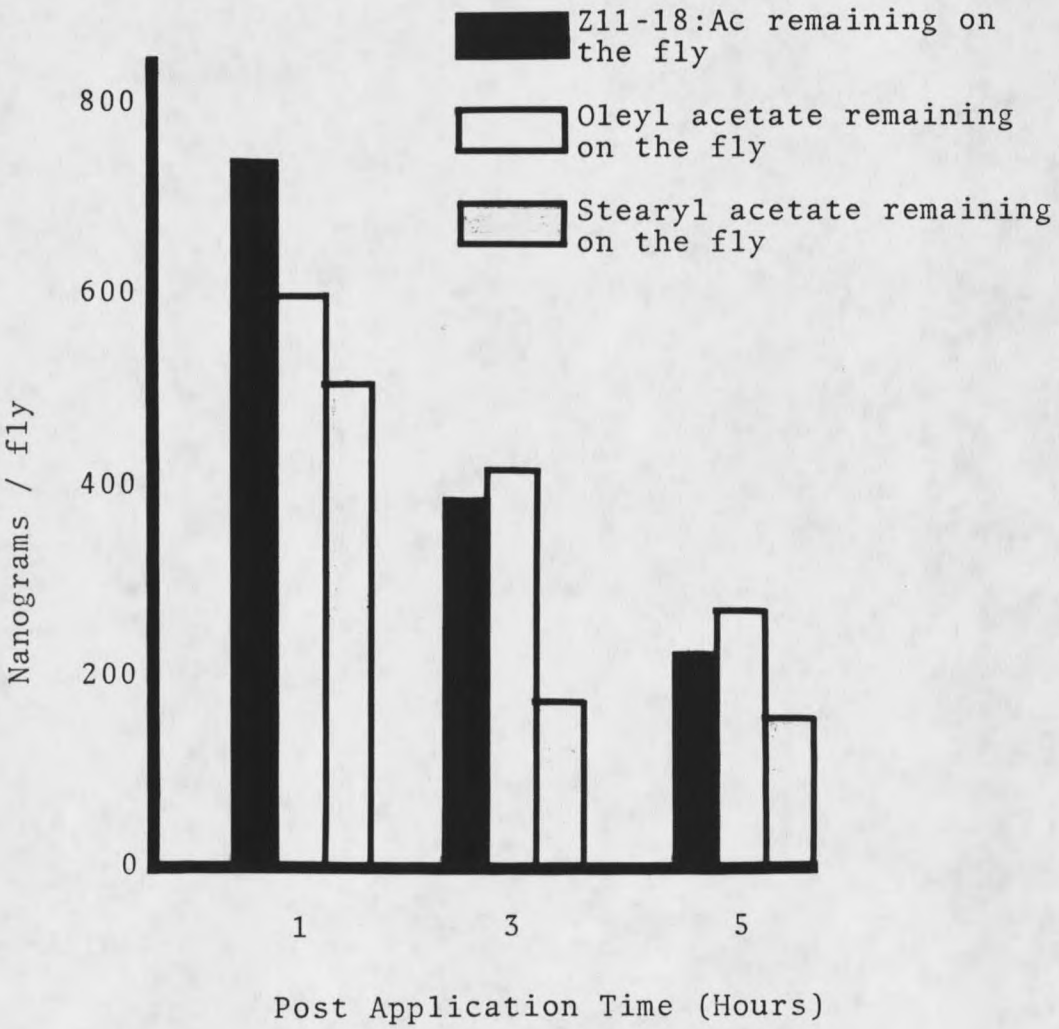

Figure 6. Application of 1100 ng (Z)-11-Octadecenyl acetate (Z11-18:Ac), 1000 ng of oleyl and stearyl acetate to 7 day old virgin female D. funebris (N=1 set of 10 flies).

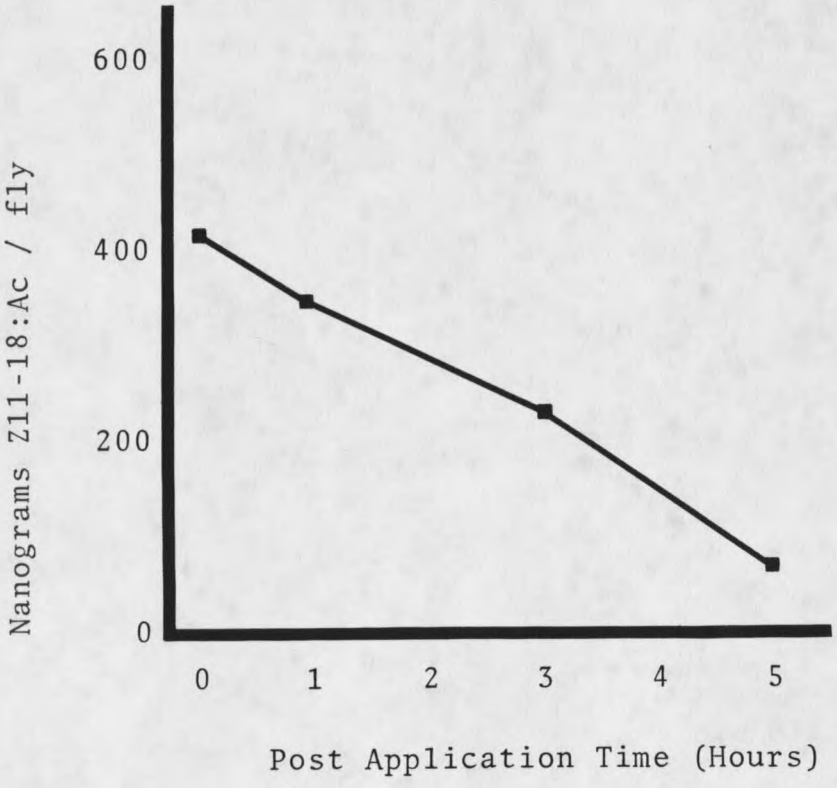

Figure 7. Application of 500 ng of (Z)-11-Octadecenyl acetate (Z11-18:Ac) to 4-5 day old virgin female D. melanogaster (N=1 set of 10 flies).

addition, holding vial rinses contained no Z11-18:Ac. Soaking the flies for 24 hours removed no additional internal Z11-18:Ac. Thus, it appeared that the ability to metabolize Z11-18:Ac was common to both species.

Bioassay Response

Identification of the aggregation pheromone began by preparing a crude hexane extract of 7 day old flies. Aging the flies to 7 days ensured that they were mature when extracted and a reproducible amount of material could be removed by extraction.

When the flies were first put into the wind-tunnel olfactometer, they tended to form tight aggregation units in the corners and on the upwind screen. An overnight holding temperature of 20° C. was used to reduce stress. This temperature kept the flies tightly aggregated. One half hour before the tests began, the temperature was increased to 24° C. and lights were turned on. This aided in the relatively uniform dispersal of the flies and also increased the frequency of flies that were in flight. While running the bioassay tests, the cage temperature was slowly increased to a final temperature of 26°C. This resulted in a better bioassay response than if a constant temperature was maintained. In response to an active preparation the number of airborne flies increased. However, when responding to an active preparation, 1/2-2/3 of the

responding flies crawled up the sides and into the vials. It became very evident within 30 seconds if a vial contained an active preparation because the number of flies crawling toward the vial increased dramatically. Once inside the vial, flies usually remained for the duration of the test. The same response behaviors were exhibited toward both fly-derived and synthetic materials. The absolute bioassay catches for any treatment varied from day to day, due primarily to the overnight holding temperature and in the number and condition of the flies in the wind-tunnel olfactometer.

Initial attempts to demonstrate an aggregation response in D. funebris were patterned after previous work with D. melanogaster (16) and D. simulans (17). The mature male and female fly (7 day old) hexane extracts along with immature male and female fly (0-24 hours) hexane extracts were bioassayed to determine the aggregation activity, as shown in Table 4. Hexane extract of mature males was clearly attractive, catching an average of 23.1 flies per test with 55% of the flies being male. Both the immature male and immature female extracts showed little activity and were not significantly different from the hexane control. Mature female extract was significantly different from the control but showed little activity. It was decided to work with extracts from mature males and determine what component(s) produced the aggregation response.

Table 4. Aggregation response of several extracts relative to the mature male *D. funebris* hexane extract.

Hexane Extract of:[a]	Relative Response[b] (N=8)
Mature Males	100*
Immature (0-24hr) Males	3
Mature Females	4*
Immature (0-24 hr) Females	-5

Hexane Control (1.3 flies/test); mature males (23.1 flies/test)

a) All extracts used at one fly equivalent

b) Relative Response = $\frac{\text{(Extract - Control)}}{\text{(Mature Male Extract - Control)}} \times 100$

* Denotes significant difference vs control at the 0.05 level

The hexane extract from 7 day old virgin male flies was fractionated using an open column of silicic acid and eluting with hexane; 5%, 10%, 25% and 50% ether-hexane; and 10% methanol/methylene chloride. The three most polar fractions showed some activity, but various combinations, when compared to the male hexane extract, would not produce an equivalent aggregation response.

Examining previous work showed that the aggregation pheromone of D. mulleri (22) contained an unidentified, volatile, polar component. To test this in D. funebris, a sample of the male hexane extract was taken to dryness under nitrogen and an equivalent amount of hexane was added back to the remaining residue. The aggregation response of the "evaporated" male hexane extract was reduced over 50% when compared to the untreated male hexane extract. Clearly, a volatile component of the male hexane extract was lost and also essential for aggregation activity. This would explain why a combination of silicic acid fractions could not account for all the activity of the male hexane extract. The combined fractions had been partially evaporated under nitrogen to achieve the proper concentration for bioassay tests.

A fraction was prepared for bioassay tests by trapping the volatile component from the male hexane extract in Tenax. In the wind-tunnel olfactometer, the Tenax volatiles produced a good response, as shown in Table 5, although not equivalent to the male hexane extract's response. A combination of the Tenax volatiles with the 5% ether-hexane fraction in bioassay produced a response equivalent to the response of the hexane extract of males. Since GC showed Z11-18:Ac to be the major component in the 5% ether-hexane fraction, Z11-18:Ac was tested with the Tenax volatiles. Tenax volatiles accounted for approximately 40%

of the male hexane extract's activity, but when added to Z11-18:Ac the response of the combination was statistically equivalent to the male hexane extract, as shown in Table 5.

Table 5. Response of *D. funebris* to the male hexane extract and two components of the extract.

Treatment[a]	Mean Bioassay Catch[b] (N=12)
Male Hexane Extract	20.7a
Tenax Volatiles from Male Hexane Extract & Z11-18:Ac (2200 ng)	21.8a
Tenax Volatiles from Male Hexane Extract	7.9b
Z11-18:Ac (2200 ng)	3.6c

a) All fly-derived fractions, extracts and synthetic compounds were used at 1 fly equivalent per test.

b) Means followed by different letters were significantly different at the 5% level (LSD).

Tenax volatiles collected from the mature female hexane extract and analyzed on the GC showed little similarity to the male Tenax extract. This could account for the minimal aggregation response of the mature female hexane extract.

Thus far, attempts to isolate and identify the component(s) in the Tenax fraction were unsuccessful. There appeared to be more than one component but reproducing results with the volatile compound(s) was difficult. A

volatile component in *D. mulleri*'s pheromone was never identified (22), and an attractive polar component in *D. virilis* (12) remained unidentified. Until a new procedure has been developed and tested, the volatile component(s) of *D. funebris*' pheromone system will remain unidentified.

CONCLUSIONS

1. (Z)-11-Octadecenyl acetate was identified as the most abundant component of the male ejaculatory bulb.
2. Z11-18:Ac was produced by males at a rapid rate during the first three days after eclosion to a level of approximately 2200 ng/fly of which approximately 1500 ng was stored in the ejaculatory bulb and approximately 800 ng was on the surface of the fly.
3. During mating the male transferred approximately 1200 ng of Z11-18:Ac to the female with over half transferred in the first five minutes of an average 16 minute mating. Most of the Z11-18:Ac was on the cuticle of the mated female and approximately 20% was in the female reproductive tract.
4. The mated female fly released approximately 5% of the 1200 ng of transferred Z11-18:Ac to the food media. They catabolized the remaining Z11-18:Ac within about 12 hours after mating. Mature virgin females catabolize Z11-18:Ac, vaccenol, vaccenic acid, stearyl acetate, and oleyl acetate.
5. Volatile, polar components from the male hexane extract along with Z11-18:Ac comprise the aggregation pheromone system in *D. funebris*.

REFERENCES CITED

1. Merrell, D.J.1951. Interspecific competition between Drosophila funebris and Drosophila melanogaster. Am. Nat. 85:159-169.

2. Ewing, A.W. 1979. Complex courtship songs in the Drosophila funebris Species Group: Escape from an evolution bottleneck. An. Behav. 27:343-349.

3. Harshman, L.G. and Hoffmann, A.A. 1987. Residual influences on fecundity is Drosophilid species. Exp. 43:213-215.

4. Shorrocks, B. and Charlesworth, P. 1980. The distribution and abundance of the British fungal-breeding Drosophila. Ecol. Ent. 5:61-78.

5. Dubinin, N.P. and Tiniakov, G.G. 1946a. Structural chromosome variability in urban and rural populations of Drosophila funebris. Am. Nat. 80:393-395.

6. Dubinin, N.P. and Tiniakov, G.G. 1946b. Inversion gradients and natural selection in ecological races of Drosophila funebris. Genetics 31:537-545.

7. Wallace, B. 1966. On the dispersal of Drosophila. Am. Nat. 100:551-563.

8. Spencer, W.P. 1935. The non-random nature of visible mutations in Drosophila. Am. Nat. 69:223-238.

9. Tantawy, A.O. and El-Wakil, H.M. 1970. Studies on natural populations of Drosophila XI. Fitness components and competition between Drosophila funebris and Drosophila virilis. Evol. 24:528-530.

10. Bauman, H. 1974a. The isolation, partial characterization, and biosynthesis of the Paragonial substances, PS-1 and PS-2, of Drosophila funebris J. Insect Phys. 20:2181-2194.

11. Baumann, H. 1974b. Biological effects of Paragonial substances PS-1 and PS-2, in females of Drosophila funebris. J. Insect Physiol. 20:2347-2362.

12. Bartelt, R.J. and Jackson, L.L. 1984. Hydrocarbon component of the Drosophila virilis (Diptera: Drosophilidae) aggregation pheromone: (Z)-10-Heneicosene. Ann. Entomol. Soc. Am. 77:364-371.

13. Bartelt, R.J., Jackson, L.L., and Schaner, A.M. 1985a. Ester components of the aggregation pheromone of Drosophila virilis (Diptera: Drosophila). J. Chem. Ecol. 11:1197-1208.

14. Bartelt, R.J., Schaner, A.M., and Jackson, L.L. 1986. Aggregation pheromone in five taxa of the Drosophila virilis species group. Physiol. Entomol. 11:367-376.

15. Bartelt, R.J., Schaner, A.M. and Jackson, L.L. Accepted. Aggregation pheromones in Drosophila borealis and Drosophila littoralis.J. Chem. Ecol.

16. Bartelt, R.J., Schaner,A.M. and Jackson, L.L. 1985b. cis-Vaccenyl acetate as an aggregation pheromone in Drosophila melanogaster. J. Chem. Ecol. 11:1747-1756.

17. Schaner, A.M., Bartelt,R.J., and Jackson, L.L. 1987. (Z)-11-Octadecenyl acetate, an aggregation pheromone in Drosophila simulans. J. Chem. Ecol. 13:1777-1786.

18. Schaner, A.M., Jackson, L.L., Graham, K.J. and Leu, R.D. Accepted. (Z)-11-Eicosenyl acetate, an aggregation pheromone in Drosophila malerkotliana. J. Chem. Ecol.

19. Schaner, A.M., Graham, K.J. and Jackson, L.L. Submitted Aggregation Pheromone Characterization and Comparison in Drosophilaananassae and Drosophila bipectinata J. Chem. Ecol.

20. Schaner, A.M., Benner, A.M., Leu, R.D. and Jackson, L.L. Submitted. Aggregation Pheromone of Drosophila mauritiana, Drosophila yakuba and Drosophila rajasekari. J. Chem. Ecol.

21. Moats, R.A., Bartelt, R.J. and Jackson, L.L. 1987. Ester and ketone components of the aggregation pheromone of Drosophila hydei. J. Chem. Ecol.13:451-462.

22. Bartelt, R.J., Schaner, A.M. and Jackson, L.L. Accepted. Aggregation pheromone components in Drosophila mulleri A chiral ester and an unsaturated ketone. J. Chem. Ecol.

23. Zawistowski,S. and Richmond, R.C. 1986. Inhibition of courtship and mating of Drosophila melanogaster by the male-produced lipid, cis-vaccenyl acetate. J. Insect Phys. 32:189-192.

24. Vander Meer, R.K., Obin, M.S., Zawistowski, S., Sheehan,K.B. and Richmond, R.C. 1986. A reevaluation of the role of cis-vaccenyl acetate,cis-vaccenol and esterase 6 in the regulation of mated female sexual attractiveness in Drosophila melanogaster. J. Insect Physiol. 32:681-686.

25. Scott,D. and Richmond, R.C. 1987. Evidence against an anti-aphrodisiac role for cis-vaccenyl acetate in Drosophila melanogaster. J. Insect Physiol. 33:363-369.

26. Beroza, M. and Bierl, B.A. 1967. Rapid determination of olefin positions in organic compounds in microgram range by ozonalysis and gas chromatography. Anal. Chem. 39:1131-1135.

27. Yates, F. 1940. The recovery of interblock information in balanced incomplete block designs. Ann. Eugen. 10:317-325.

DATE DUE

MAY 1 5 199?			