(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

	Page
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	vii
INTRODUCTION	1
Objectives	4
MATERIALS AND METHODS	5
Ejaculatory Bulb Removal and Analysis	5
Extraction of Whole Flies	6
Formation of the Ejaculatory Bulb Compound with Age	7
Transfer During Mating	7
Female Reproductive Tract Removal	7
Ejaculatory Bulb Compound Loss by the Female	8
Application of Compounds to Virgin Females	8
Collection of Volatiles from Hexane Extracts	9
Bioassays	9
RESULTS AND DISCUSSION	11
Identification	11
Formation	11
Transfer During Mating	13
Catabolism	17
Application of Z11-18:Ac	19
Application of Vaccenic Acid	22
Application of Vaccenol	24
Application of Similar Acetates	25
Application of Z11-18:Ac to *D. melanogaster*	25
Bioassay Response	28
CONCLUSIONS	34
REFERENCES CITED	35

LIST OF TABLES

Table		Page
1.	Drosophila species with identified aggregation pheromones	2
2.	A comparison of the amounts of Z11-18:Ac sequentially extracted from *D. funebris* and *D. melanogaster* mated females	15
3.	Recovery of Z11-18:Ac from mated and virgin *D. funebris*	17
4.	Aggregation response of several extracts relative to the mature male *D. funebris* hexane extract	30
5.	Response of *D. funebris* to the male hexane extract and two components of the extract	32

LIST OF FIGURES

Figure		Page
1.	(Z)-11-Octadecenyl acetate (Z11-18:Ac) in male *D. funebris* with age	12
2.	Seven day old *D. funebris* matings with the subsequent removal of the ejaculatory bulb, reproductive tract and hexane soak of the fly	14
3.	Loss of (Z)-11-Octadecenyl acetate (Z11-18:Ac) from mated *D. funebris* females with time	18
4.	Application of 1100 ng of (Z)-11-Octadecenyl acetate (Z11-18:Ac) to 7 day old virgin female *D. funebris*	20
5.	Application of 2500 ng of vaccenic acid and 1000 ng of vaccenol to mature *D. funebris* females	23
6.	Application of 1100 ng of (Z)-11-Octadecenyl acetate (Z11-18:Ac), 1000 ng of oleyl and stearyl acetate to 7 day old virgin female *D. funebris*	26
7.	Application of 500 ng of (Z)-11-Octadecenyl acetate (Z11-18:Ac) to 4-5 day old virgin female *D. melanogaster*	27

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

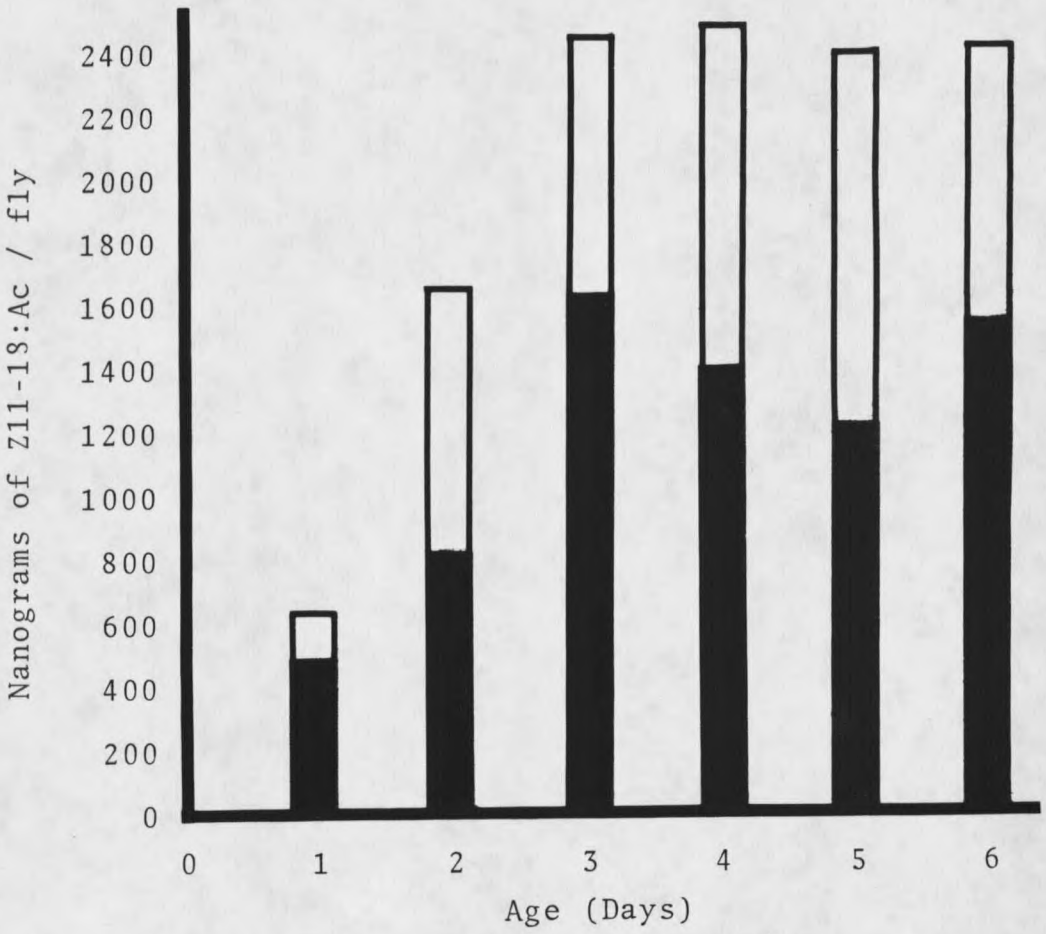

Figure 1. (Z)-11-Octadecenyl acetate (Z11-18:Ac) in male D. funebris with age. Shaded areas represent Z11-18:Ac in the ejaculatory bulb while clear areas represent Z11-18:Ac on the rest of the fly (N=2 sets of 3 flies).

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

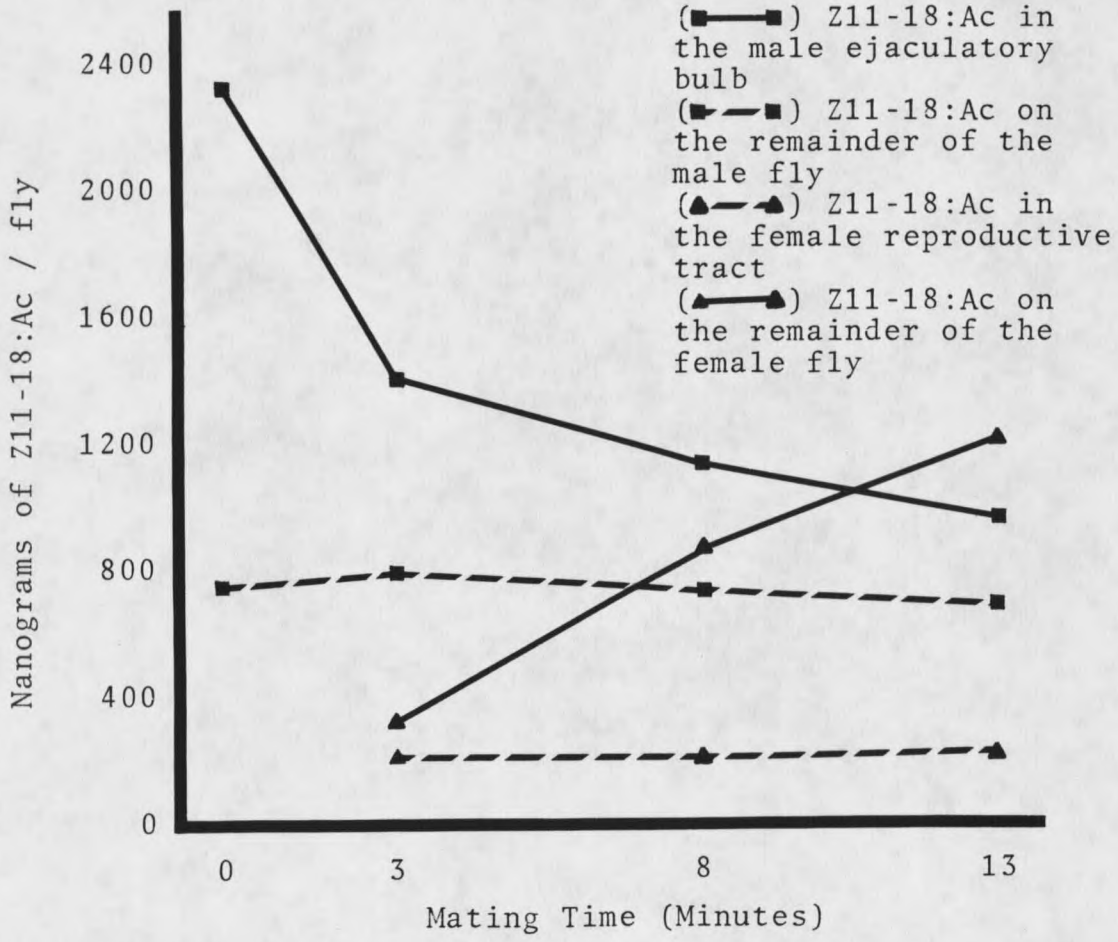

Figure 2. Seven day old D. funebris matings with the subsequent removal of the ejaculatory bulb, reproductive tract and hexane soak of the fly. (N=3 sets of 3 flies)

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