Archives of Insect Biochemistry and Physiology 29:87–97 (1995)

Sex- and Age-Related Changes in the Biophysical Properties of Cuticular Lipids of the Housefly, *Musca domestica*

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We examined the biophysical properties of cuticular lipids isolated from the housefly, *Musca domestica*. Melting temperatures (T_m) of surface lipids isolated from female houseflies decreased from 39.3°C to 35.3°C as the females attained sexual maturity and produced sex pheromone, whereas those prepared from males did not change with age. Lipids melted over a 10–25°C temperature range, and their physical properties were a complex function of the properties of the component lipids. The T_m of total cuticular lipids was slightly below that of cuticular hydrocarbons (HC), the predominant lipid fraction. Hydrocarbons were further fractionated into saturated, unsaturated, and methyl-branched components. The order of decreasing T_m was total alkanes > total HCs > methyl-branched alkanes > alkenes. For 1-day-old flies, measured T_ms of hydrocarbons were 1.3–5.5°C lower than T_ms calculated from a weighted average of T_ms for saturated and unsaturated components. For 4-day-old flies, calculated T_ms underestimated T_m by 11–14°C. © 1995 Wiley-Liss, Inc.

Key words: cuticular lipid, housefly, hydrocarbon, methyl-branched, pheromone, unsaturation

INTRODUCTION

Epicuticular lipids of insects and other land arthropods serve two important biological functions. In all terrestrial life stages, these lipids provide the primary passive barrier to evaporative water loss (Edney, 1977; Hadley, 1994). In many species, certain components also play a role in chemical communication, either intra- or interspecifically (Blomquist et al., 1993; Howard, 1993). The multiple roles of cuticular lipids suggest that differences in composition related to one function may have secondary effects upon other functions. For example, Markow and Toolson (1990) found that, in the desert fruit fly

Acknowledgments: This research was supported by NSF grants IBN-9317471 to A. Gibbs and IBN-9220092 to G.J. Blomquist.

Received September 15, 1994; accepted December 28, 1994.

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Drosophila mojavensis, thermal acclimation of cuticular hydrocarbons affected the relative amounts of putative male sex-recognition pheromones, with correlated negative effects on mating success. Conversely, it could be the case that synthesis of semiochemicals might increase the permeability of the lipid barrier and thereby adversely affect water balance.

The cuticular lipids of houseflies, *Musca domestica*, are perhaps the most thoroughly characterized of all insects. Over 150 different cuticular lipid components have been found and identified in this species. The majority of these are hydrocarbons (Nelson et al., 1981; Blomquist et al., 1993). One of these, (*Z*)-9-tricosene, is characteristic of reproductively mature females, in which it may constitute as much as 10% of the total surface lipid. (*Z*)-9-tricosene is the primary constituent of the female sex pheromone, although other compounds, including methylalkanes and nonhydrocarbons, potentiate its effects on male reproductive behavior (Carlson et al., 1971; Adams and Holt, 1987; Blomquist et al., 1993).

One might predict that deposition of the cuticular sex pheromone would affect water relations of female houseflies. The reason is that the waterproofing properties of cuticular lipids are believed to depend largely on their biophysical properties, in particular their phase behavior (Wigglesworth, 1945; Noble-Nesbitt, 1991). Unsaturation and methyl-branching both decrease the melting temperatures (T_m) of pure cuticular hydrocarbons (Gibbs and Pomonis, in press) and thus could increase water loss rates. Because the housefly pheromone is abundant and contains both unsaturated and methyl-branched components, cuticular lipids of reproductively mature females might melt at reduced temperatures. Thus, one goal of this study was to determine whether or not reproductively mature females exhibit differences in the physical properties of their surface lipids compared to males and newly emerged females. An additional objective was to understand which aspects of cuticular lipid composition (lipid class, chain length, unsaturation, and methyl-branching) are important in determining overall physical properties. We found that melting temperatures of cuticular lipids of female houseflies decreased by 4°C as the flies matured, whereas those of males did not change. The properties of the total cuticular lipid mixture were a complex function of the component lipids. Polar lipids and unsaturated and branched hydrocarbons tended to reduce T_m. Melting temperatures of saturated hydrocarbons were slightly higher than those of total cuticular hydrocarbons.

MATERIALS AND METHODS

Houseflies

Musca domestica (1958 Fales strain T-II) were obtained from the Biology Section, S.C. Johnson and Sons (Racine, WI) as pupae. This strain has previously been used in studies of cuticular pheromone biosynthesis (e.g., Ahmad et al., 1989; Blomquist et al., 1994), thereby allowing us to make direct comparisons to previous analyses. Flies were allowed to emerge in the laboratory and were maintained as separate sexes at room temperature (~27°C) for 1 or 4 days after eclosion. One-day insects were 0–24 h postemergence, and 4-day insects were 72–96 h postemergence.

Lipid Extraction and Fractionation

Cuticular lipids were isolated as described by Nelson et al. (1981). All lipid samples were stored at –20°C under N_2 until analyzed. Three different groups of houseflies were analyzed:

Group I. Males and females were analyzed 1 day and 4 days posteclosion. Each treatment group was divided into three subsamples of 30 flies, and each sample was extracted separately. Total cuticular lipids were extracted from intact flies by immersion in hexane for 10 min, followed by a 1 min hexane extraction. Cuticular hydrocarbons in the combined extracts were separated from polar lipids by column chromatography on Bio-Sil A (Nelson et al., 1981; Dillwith et al., 1983). Group I samples were prepared in December, 1992.

Group II. In order to determine how different lipid components affect overall lipid physical properties, total cuticular lipids were isolated from one hundred 1-day- and 4-day-old flies of each sex. Polar and nonpolar (hydrocarbon) fractions were separated by Bio-Sil A chromatography. Total HCs were then fractionated into saturated and unsaturated components using $AgNO_3$-silica gel chromatography. Saturated HCs were treated further by molecular sieving to isolate methyl-branched alkanes. For both total surface lipids and hydrocarbons, T_m values were in the range determined for group I houseflies, so these data were pooled in later analyses. This group (prepared in April, 1993) did not include replicate subsamples.

Group III. The cuticular alkene fraction from 1-day-old females in Group II was found to be contaminated. We therefore isolated cuticular hydrocarbons from a third set of houseflies in April, 1994. Total HCs, total alkanes, and alkenes were isolated from group I and group II females. An analysis of variance revealed that these differences were statistically significant ($P < 0.05$). Total alkanes also exhibited consistently higher T_m values than alkanes from 1-day-old females in group II. The explanation for the intergroup variability is unclear. We have previously noticed differences in viability among different shipments of pupae, presumably due to variation in rearing or shipping conditions. We decided to err on the side of caution and excluded group III from further analyses, except for the purpose of estimating T_m of alkenes from 1-day-old females. We note, however, that we observed the same patterns of differences between hydrocarbon classes in group III flies as in group II.

Lipid Analyses

Lipid physical properties were analyzed using a Perkin-Elmer Systems 2000 Fourier transform infrared spectrometer, essentially as described by Gibbs and Crowe (1991). Ten to 100 μg of lipid were dissolved in hexane and spotted onto an infrared-transparent CaF_2 window. The sample was placed in a Peltier device temperature controller, which was then cooled to at least 20°C below the solid-liquid phase transition temperature. The temperature was ramped up at 2°C intervals until the lipids were completely melted. In order to avoid condensation of water vapor and possible artefacts associated with lipid oxidation, the sample chamber was purged continuously with nitrogen. Five infrared spectra were averaged at each temperature. The progress of lipid melting was determined from the $-CH_2-$ symmetric stretching absorbance peak, which shifts from ~2,849 cm^{-1} in the solid state to ~2,853 cm^{-1} as

lipids melt (Crowe et al., 1989; Gibbs and Crowe, 1991). The location of this peak provides an index of the relative amount of *trans-gauche* isomerization around carbon-carbon bonds of hydrocarbon chains, as occurs when lipids melt (Blazyk and Rana, 1987).

Gas chromatographic (GC) analyses of lipid composition were performed using a Hewlett-Packard 5890A gas-liquid chromatograph with a 30 m × 0.32 mm DB-5 capillary column programmed from 160 to 300°C at a rate of 4°C/min and maintained at the final temperature for 15 min.

Data Analysis

We fitted plots of temperature vs. peak frequency (cm^{-1}) to sigmoidal equations. The lipid melting temperature (T_m; midpoint of the phase transition) and the width of the phase transition (ΔT; the difference between the 95% and 5% melted points of the fitted curve) were calculated from this equation. Statistical analyses were performed using the data analysis package in Microsoft Excel. In order to estimate the repeatability of T_m and ΔT measurements, most lipid samples were divided into two portions, and FTIR analyses were performed on each portion separately. The mean value of T_m or ΔT obtained for a given sample was used as the dependent variable in statistical tests. This was done because the replicate measurements were not statistically independent of each other.

RESULTS

Representative melting curves for total cuticular lipids are presented in Figure 1. Lipids typically melted over a 10–25°C range. For both total cuticular lipids and for hydrocarbons, T_m and ΔT were not correlated, nor did age or

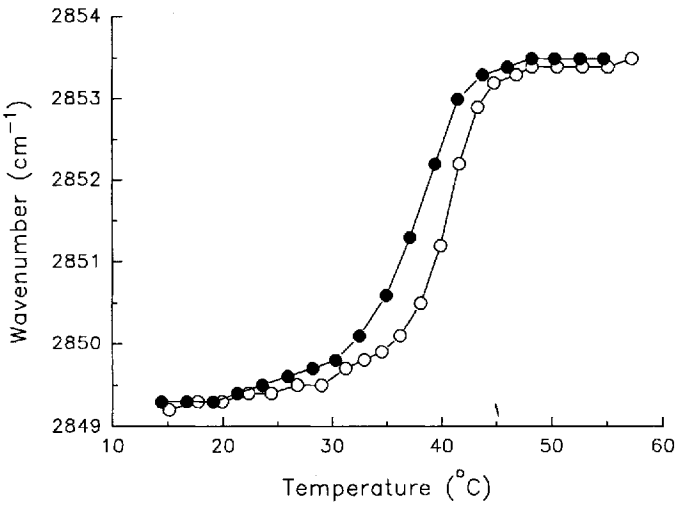

Fig. 1. Representative melting curves for total cuticular lipids from female houseflies. Open circles: one day old, T_m = 39.2°C, ΔT = 15.4°C. Filled circles: four days old, T_m = 35.3°C, ΔT = 23.2°C.

sex significantly affect ΔT (ANOVA, $P > 0.10$). In other words, samples with higher T_m values did not tend to melt over a relatively broad or narrow range of temperatures. Because FTIR is a relatively new technique with respect to the analysis of cuticular lipids, we wanted to assess the repeatability of our T_m and ΔT estimates. We therefore performed two separate FTIR analyses on each total lipid and total hydrocarbon sample, as well as several HC fractions. The mean absolute value of the difference between replicate measurements of T_m was 1.64°C (±0.18°C S.E.; range = 0–5.4°C; n = 38). Replicate determinations of ΔT for total lipids and hydrocarbons were more variable; the mean absolute difference was 3.1°C (±0.4°C S.E.; range = 0–9.0°C; n = 34). These values provide an estimate of the repeatability of measurements of T_m and ΔT.

Melting temperatures of total cuticular lipids of 1-day-old houseflies were 37–41°C and did not differ between males and females (t-test, $P > 0.1$). Melting points of surface lipids from males did not change with age ($P > 0.3$), whereas those of females decreased from 39.3°C to 35.3°C (Fig. 2). Cuticular lipid T_ms of 4-day-old females were significantly lower than those of 4-day-old males and 1-day-old females ($P < 0.005$ for both comparisons).

Changes in cuticular HC properties resembled those of total cuticular lipids (Fig. 2). Melting points decreased from 39.8°C to 37.4°C as female houseflies aged, and T_ms for 4-day-old females differed significantly from those of 1-day-old females and 4-day-old males (t-test, $P < 0.01$ and $P < 0.05$, respectively). Hydrocarbon T_ms were slightly (0.5–2.1°C) higher than those of total cuticular lipids from flies of the same age and sex. An analysis of variance indicated that this difference was statistically significant ($P < 0.005$).

In order to gain more insight into the chemical basis for differences in T_m, we separated one cuticular lipid sample (group II flies) from each age and sex into polar and nonpolar (hydrocarbon) fractions. The results of gas chromatographic analyses of HC composition are presented in Table 1. The hydrocarbons were then subdivided into alkenes and total alkanes, from which we then isolated methylalkanes. The results of FTIR analyses of the lipid fractions are presented in Table 2. For each sex and age, the order of decreasing T_m was saturated HC > total HC > total cuticular lipid > methylalkanes > alkenes. Polar lipids varied widely in T_m (11–34°C), but because they are minor constituents we did not investigate them further.

DISCUSSION

In this study, we examined sex- and age-dependent differences in the physical properties of cuticular lipids of houseflies as well as the structural basis for lipid phase behavior. Our results are addressed from two perspectives: physiological and biophysical. The physiological point of view involves three biologically oriented questions: What are the melting points of housefly surface lipids? Do cuticular lipids melt at physiologically relevant temperatures? How does T_m depend on age and sex? The biophysical question is this: How do structural changes in cuticular lipids affect lipid physical properties? Given that surface lipid phase behavior does affect cuticular permeability, the answer to this question will have implications for understanding the functional

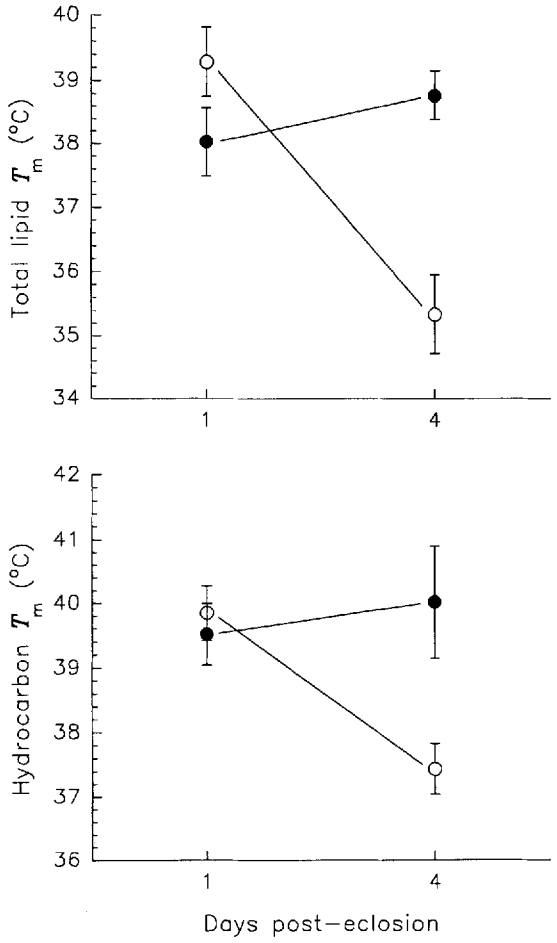

Fig. 2. Age-dependent changes in T_m for cuticular lipids isolated from females (open circles) and males (filled circles). For a given sex and age, two measurements were made on each of four samples. Data represent means (±1 S.E.) of average T_m for each sample (i.e., means of means). **Upper panel:** Total cuticular lipids. **Lower panel:** Hydrocarbons.

physiological significance of cuticular lipid variation in many other terrestrial arthropods besides houseflies. We must stress at the outset, however, that a detailed understanding of the relationship between cuticular permeability and surface lipid properties is still lacking, although several studies have provided experimental evidence consistent with the idea that the two are related (Beament, 1945; Toolson et al., 1979; Machin and Lampert, 1990).

Melting temperatures of housefly cuticular lipids were 35–40°C. This is a rather high though not necessarily an irrelevant temperature range for this species. However, we must point out that the T_m is simply the midpoint of the phase transition. Surface lipids melted over a range of 10–25°C (i.e., they often started melting below 30°C, which is certainly a reasonable environ-

TABLE 1. Composition of Cuticular Hydrocarbons of Group II Houseflies*

Chain length	Day-1 males	Day-4 males	Day-1 females	Day-4 females
n-Alkenes				
23	0.0	0.0	0.0	10.6 ± 0.7
27	2.4 ± 0.2	46.0 ± 0.4	1.3 ± 0.1	21.6 ± 1.0
29	2.1 ± 0.1	7.2 ± 0.5	2.9 ± 0.2	4.4 ± 0.2
31	1.5 ± 0.2	0.7 ± 0.1	0.0	0.0
Total	6.0	53.9	4.2	36.6
n-Alkanes				
23	0.8 ± 0.1	4.1 ± 0.1	0.7 ± 0.1	14.8 ± 0.9
25	2.7 ± 0.2	20.5 ± 0.5	2.4 ± 0.3	8.8 ± 0.4
26	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.0
27	13.7 ± 1.1	14.7 ± 0.5	14.1 ± 1.6	9.2 ± 0.2
29	3.1 ± 0.1	1.1 ± 0.1	6.6 ± 0.7	6.2 ± 0.2
30	0.0	0.0	2.9 ± 0.5	0.8 ± 0.6
31	3.2 ± 0.2	0.0	1.4 ± 0.2	1.2 ± 0.2
Total	24.1	40.9	28.8	41.0
Methylalkanes[a]				
27	7.1 ± 0.4	1.0 ± 0.1	6.2 ± 0.3	5.4 ± 0.2
28	1.5 ± 0.1	0.0	4.5 ± 0.6	2.4 ± 0.1
29	28.6 ± 0.8	1.3 ± 0.1	22.0 ± 1.4	8.9 ± 0.4
30	8.3 ± 0.4	0.7 ± 0.2	8.0 ± 0.1	3.3 ± 0.6
31	15.4 ± 1.1	1.2 ± 0.1	15.7 ± 1.1	1.7 ± 0.1
33	4.9 ± 0.7	0.6 ± 0.1	5.1 ± 1.6	0.7 ± 0.1
35	4.1 ± 0.7	0.0	5.5 ± 2.0	0.0
Total	69.9	4.8	67.0	22.4

*Data are expressed as mean percentage of total hydrocarbons for three GC analyses (±S.D.).
[a]Numbers indicate chain length; components contained one or two methyl branches.

mental temperature). Unlike the case for the grasshopper, *Melanoplus sanguinipes*, in which higher melting point lipids melt over a narrower range in temperature (Gibbs et al., 1991), T_m and ΔT were not significantly correlated in houseflies.

The only change in lipid properties which we observed was a decrease in T_m as females aged. This may result in increased water loss, but a complicat-

TABLE 2. Melting Temperatures for Hydrocarbons Isolated From Group II Houseflies

Lipid fraction	Day-1 males	Day-4 males	Day-1 females	Day-4 females
Alkenes	10.6	12.2	6.8[a]	–0.2
Alkanes	48.2	40.5	43.2	41.5
Methylalkanes	34.2	22.6	27.5	29.9
Total HCs	40.4	39.4	40.4	36.8
HCs predicted	45.9	25.1	41.7	25.1

*Each value indicates a single melting point determination. Predicted T_ms for total hydrocarbons were calculated as the weighted average of the T_m values for alkenes and alkanes isolated from the same samples.
[a]Mean of T_ms for three alkene samples from group III houseflies (range = 6.4–7.2°C). Total hydrocarbons and alkanes from these files melted 2–3°C higher than did those isolated from group I and II.

ing factor is lipid density. Mature females contain greater amounts of surface lipid than males (Nelson et al., 1981), but are also larger and thus have a greater surface area. We did not quantify the amounts of surface lipid on our flies, because adding the necessary internal standard would have made it impossible to obtain accurate FTIR data. Depending on the relative effects of qualitative and quantitative changes in cuticular lipids, we speculate that pheromone production may adversely affect water balance in mature females. We are unaware of any studies documenting age- or sex-related changes in water loss rates of *M. domestica*, and certainly none correlating water loss rates with surface lipid composition or quantity are known.

We did not measure water loss rates for two reasons. First, the strain of houseflies used had been maintained in laboratory culture for over 30 years. We chose the T-II strain because it has been the subject of several studies of pheromone biosynthesis (Ahmad et al., 1989; Blomquist et al., 1993, 1994). However, inbreeding or adaptation to long-term laboratory culture may have affected water loss rates in ways unrelated to cuticular lipids (Toolson and Kuper-Simbrón, 1989). Second, we have observed differences in viability between batches of flies shipped at different times of the year as well as between surface lipids of groups I and III in this study. Unknown differences in treatment before the pupae reached the lab might have affected other physiological characters related to water loss.

If lipid physical properties do affect cuticular permeability, one would like to understand the structural basis for differences in T_m. Are cuticular lipid properties a simple weighted average of the properties of the component lipids, or do interactions between different components affect overall lipid phase behavior? Which structural changes are most important? Candidates include lipid class, chain length, methyl-branching, and unsaturation. With respect to lipid class, cuticular lipids of *M. domestica* are primarily composed of hydrocarbons (Nelson et al., 1981). Total surface lipids melted slightly below hydrocarbons, reflecting that total lipids also contained small amounts of the low T_m polar lipid fraction. The predominance of hydrocarbons, and the fact that age- and sex-related changes in T_m were similar for total lipids and hydrocarbons, indicates that hydrocarbon properties are the major determinant of surface lipid phase behavior in *M. domestica*.

If hydrocarbons dominate total surface lipid properties, what determines hydrocarbon properties? Because only one of our treatment groups, the 4-day-old females, differed from any of the others, we cannot make any inferences about the effects of particular compounds on T_m. Instead, our analysis will focus on broad patterns of cuticular lipid structure and their effects on lipid properties. We found that T_m values for hydrocarbons fractionated from all four sex and age treatments followed the same sequence: saturated > total > methyl-branched > unsaturated (Table 2). Chain length was not correlated with T_m; the rank order of mean chain lengths for HC classes was methyl-alkanes > *n*-alkenes > *n*-alkanes (Table 3). Chain length has received the most attention in studies of temperature adaptation and water balance (Hadley, 1977, 1978; Toolson and Hadley, 1977, 1979; Hadley and Schultz, 1987), but the current study and a study of thermal acclimation of *M. sanguinipes* (Gibbs

TABLE 3. Mean Chain Lengths for Cuticular Hydrocarbons of Houseflies

Lipid fraction	Day-1 males	Day-4 males	Day-1 females	Day-4 females
Alkenes	28.7	27.3	28.4	26.1
n-Alkanes	27.6	25.6	27.7	25.6
Methylalkanes	30.0	29.7	30.1	28.8
Overall length	29.3	26.6	29.3	26.5

and Mousseau, 1994) indicate that length alone cannot explain differences in cuticular lipid properties.

Cuticular hydrocarbons of older flies were much more unsaturated; males had over 50% alkenes (Table 1). Because alkenes melted at much lower temperatures than other HC fractions (Table 2), one would expect surface lipid T_m to be lower in older flies of both sexes. However, T_m declined only in females and only by 4°C. In order to investigate the effects of unsaturation on lipid properties, we calculated the predicted T_m for total cuticular hydrocarbons from the weighted average of T_ms of the saturated and unsaturated components. Our simple linear model predicted T_m values for 1-day-old flies reasonably well but underestimated T_m for older flies by more than 10°C (Table 2). Our interpretation is that, although alkenes alone may exhibit low T_ms, they have relatively little effect on bulk lipid properties.

The next question to be addressed is why T_m decreased with age in females but not in males (Fig. 2). Both sexes exhibited broadly similar changes in the relative proportions of HC classes; methylalkane levels declined as alkene and *n*-alkane levels rose (Table 1). Unsaturation alone cannot explain the decline in T_m, since lipids in 4-day-old males were more unsaturated more than any others. Chain length may be partly responsible; 4-day-old females had the shortest chain length hydrocarbons for each class (Table 3)—differences which should result in lower T_ms. Chain length cannot be the entire explanation, however; 4-day-old males had the second shortest chain lengths in each HC class, yet their T_m values were not different from those of 1-day-old flies (Fig. 2). This leaves methyl-branching, which has been found to reduce T_m significantly in *M. sanguinipes* (Gibbs and Mousseau, 1994). Methylalkanes were almost absent from 4-day-old males, a factor that would tend to offset the T_m-lowering effects of shorter chain lengths and greater unsaturation. Methylalkane levels were also reduced in 4-day-old females, but not by as much (Table 1). It is important to remember that methylalkanes are components of the female sex pheromone (Adams and Holt, 1987; Blomquist et al., 1993), so females may need to maintain higher levels than males.

A critical question remains unanswered: What is the biological significance of these differences? We note that the 4°C decrease in T_m as female *M. domestica* attained reproductive maturity is greater than the change in T_m observed for acclimation of *M. sanguinipes* to fall and summer conditions (Gibbs et al., 1991; Gibbs and Mousseau, 1994). One must keep in mind that the relationship between cuticular permeability and lipid amounts and physical properties remains poorly understood, but the current results may have interesting implications. Major components of the female sex pheromone are

unsaturated or methyl-branched, changes which tend to lower T_m. Thus, a potential consequence of pheromone production may be an increase in cuticular transpiration (i.e., reproductive success might exert a cost in survival under desiccating conditions). Markow and Toolson (1990) have provided evidence that the converse may be true; acclimation of male *Drosophila mojavensis* to higher temperatures results in reduced mating success, apparently mediated by changes in cuticular pheromones. We speculate that interacting selective pressures on reproduction and survival may play a role in the evolution of cuticular lipids.

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