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# Cuticular pheromones and water balance in the house fly, *Musca domestica*

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## Abstract

Epicuticular lipids serve two major roles in insects. Their waterproofing properties are crucial to survival in terrestrial environments, and they serve as contact pheromones in a wide array of taxa. Both functions may be affected by the physical properties of the surface lipids. This provides the opportunity for natural selection on water conservation, mediated by lipid phase behavior, to interact with and perhaps conflict with sexual selection on communication and mate recognition. We used the common house fly, *Musca domestica*, as a model for these interacting selective forces. Male house flies preferred female models treated with a high melting-point lipid mixture, suggesting that sexual and natural selection may both act to favor longer-chain, more saturated hydrocarbons. However, higher melting points did not result in lower rates of water loss. We propose a working model in which phase separation between the unsaturated female pheromone and saturated hydrocarbons results in areas of melted, pheromone-rich lipids and regional variation in cuticular permeability.

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**Keywords:** Cuticular lipid; House fly; Hydrocarbon; *Musca domestica*; Phase separation; Pheromone; Tricosene; Water loss

## 1. Introduction

Epicuticular lipids provide the primary barrier to evaporative water loss from insects and are a key feature allowing their ecological success. Many insect taxa also use components of the surface lipids as contact pheromones (Howard, 1993). For example, cuticular hydrocarbons serve as colony recognition cues in bees (Page et al., 1991; Breed et al., 1995), wasps (Singer, 1998) and ants (Liang and Silverman, 2000; Wagner et al., 2000). Sex-specific differences in lipid com-

position are involved in mate recognition in other insects, including *Drosophila* (Ferveur, 1997), cockroaches (Schal et al., 1990) and beetles (Peschke and Metzler, 1987).

The dual roles played by cuticular hydrocarbons suggest the possibility for interacting, if not conflicting, selective pressures on the composition of the surface lipids. Proper waterproofing depends on the presence of solid lipids, as gel-phase lipids are less permeable than melted lipids (Rourke and Gibbs, 1999; Gibbs, 2002). On the other hand, contact pheromones embedded in a solid lipid matrix may be inaccessible to chemoreceptors of other individuals. Information transfer may be enhanced when pheromones are in a mobile lipid phase, but at a cost of greater cuticular water loss.

The presence of lipid pheromones may in turn

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affect cuticular permeability. In *D. melanogaster*, major constituents of the surface lipids ((*Z*)-7-tricosene and (*Z*)-7-pentacosene in males, (*Z,Z*)-7,11-heptacosadiene in females) are also major pheromone components (Antony and Jallon, 1982; Ferveur, 1997). Unsaturated hydrocarbons such as these have low melting points ( $T_m$ ) and will tend to lower the overall  $T_m$  of the surface lipids (Gibbs and Pomonis, 1995). Thus, improved communication may come at a cost of increased water loss and reduced survival in dry conditions.

The common housefly, *Musca domestica* L. (Diptera: Muscidae) is an ideal species in which to examine hydrocarbon-mediated interactions between communication and water conservation. Between ecdysis and 4 days in age, female houseflies attain reproductive maturity and deposit large quantities (up to 10% of the total lipid) of (*Z*)-9-tricosene, the major pheromone component involved in mate recognition (Carlson et al., 1971). Pure tricosene melts at 0 °C (Gibbs and Pomonis, 1995), and deposition of the pheromone is associated with a reduction in the  $T_m$  of the total surface lipid (Gibbs et al., 1995). The lipid composition of male houseflies also changes with age, but the lipid  $T_m$  does not. In addition, male houseflies will court and attempt to copulate with dead female models (or even tacks or knots of string) that have been treated with pheromone-containing lipids (Adams et al., 1995). Thus, one can investigate how the lipid environment in which pheromones are embedded modulates the behavioral effects of the pheromone.

This study addresses two major issues. First, does pheromone production by female houseflies result in increased rates of water loss? Because  $T_m$  decreases with age in females as they deposit pheromone on the cuticle, but not in males (Gibbs et al., 1995), we predicted that female houseflies would lose water more rapidly as they matured, whereas water loss rates (WLR) of males would not change with age. Second, we examined the effects of the physical state of the surrounding lipids on pheromone reception by males. Specifically, we hypothesized that males would preferentially court female models with (*Z*)-tricosene in a melted rather than solid lipid environment. This would indicate that natural selection for higher  $T_m$  values and reduced water loss and sexual selection for lower  $T_m$  values and improved mate recognition may act in opposition on cuticular lipids. Contrary to these hypotheses, our results

suggest that fine-scale variation in the hydrocarbon milieu across the cuticle may enhance pheromone reception in a high- $T_m$  hydrocarbon environment, with unclear effects on cuticular permeability.

## 2. Methods and materials

### 2.1. Houseflies

Pupae from the Fales 1958 T-II strain were obtained from S.C. Johnson and Co. (Racine, WI). This strain has been used previously for studying cuticular lipids and pheromone synthesis (e.g. Blomquist et al., 1984, 1994; Tillman-Wall et al., 1992; Schal et al., 2001). Emerging adults were separated by sex and placed in plexiglass cages with a cloth sleeve covering one end. Flies were provided with water and a mixture of dry milk and sucrose.

### 2.2. Lipid extraction and analyses

Cuticular lipids were isolated from frozen adult houseflies by immersion in hexane for 10 min. The hydrocarbon composition was determined by capillary gas chromatography, using a 30 m × 0.32 μm DB-5 column in a Hewlett-Packard 5890A gas chromatograph. Peaks were identified by comparison with *n*-alkane standards and the hydrocarbon composition determined previously for this strain (Gibbs et al., 1995). To quantify lipid amounts, we added 2.5 μg of an internal standard (*n*-docosane) to each sample. For one series of experiments, methyl-branched alkanes were isolated from lipids of 4-day-old females using molecular sieves.

Lipid melting temperatures were determined using Fourier transform infrared (FTIR) spectroscopy, as described previously (Gibbs and Crowe, 1991). Ten to fifty micrograms of lipid was dissolved in a small volume of hexane and deposited on a CaF<sub>2</sub> window. After the solvent had evaporated, the window was placed in a temperature-controlled cell holder. The sample temperature was increased in increments of ~2 °C, from ~20 °C below the melting point to ~20 °C above. The progress of lipid melting was determined from the frequency of the -CH<sub>2</sub>-symmetric stretching absorbance peak, which increases from ~2850 to 2854 cm<sup>-1</sup> as lipids melt (Gibbs and Crowe, 1991). Plots of frequency against temperature were sigmoidal, and the  $T_m$ , defined as the midpoint of

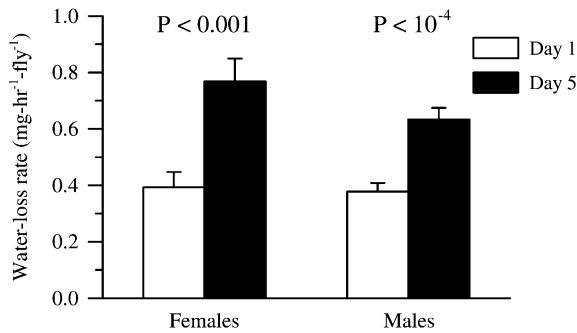


Fig. 1. Effects of age on WLR of houseflies. Means ( $\pm$ S.E.;  $n=15-17$ ).

the lipid phase transition, was calculated from a fitted logistic regression.

### 2.3. Water loss rates

Rates of water loss from individual houseflies were measured at 25 °C using a flow-through respirometry system (Sable Systems, Las Vegas, NV), as described previously (Gibbs et al., 1998; Gibbs and Matzkin, 2001). Flies were placed in 5-ml chambers, and dry, CO<sub>2</sub>-free air was pumped through the chambers at 100 ml min<sup>-1</sup>. WLR were calculated from the humidity of the downstream air, using a Licor LI-6262 CO<sub>2</sub>/humidity sensor. Identical respirometry conditions were used to measure metabolic rates as carbon dioxide production.

### 2.4. Behavioral assays

Five-day-old males were used for all behavioral assays. Each male was provided with a choice between two dead female models, each of which was treated with a different lipid mixture. The females were frozen less than 24 h after eclosion, to ensure that they did not contain endogenous pheromone. Surface lipids were extracted by 10-min immersion in hexane. Pairs of the extracted models were placed in a clear plastic arena (2×5×2 cm<sup>3</sup> high) and glued near each end with a small amount of model cement. After allowing at least 24 h for the glue to set and for fumes to disperse, the models were used in experiments.

For behavioral assays, 40 μg of a given lipid mixture was dissolved in a small volume of hexane and deposited on the upper surface of a female model. Each mixture contained 4 μg of (Z)-9-

tricosene (Sigma Chemical Company, St. Louis, MO), with the remainder consisting of either high- $T_m$  or low- $T_m$  alkanes. Care was taken to deposit lipids on all parts of the body. After the hexane evaporated, a male housefly was lightly anesthetized with CO<sub>2</sub> and placed in the chamber. After a 15-min recovery period, the fly's behavior was videotaped for 20 min using a camera mounted above the chamber. Most males actively explored their chambers and soon encountered the female models. They probed the models with their tarsi and sometimes attempted copulation. These mating strikes were clearly visible (Adams and Holt, 1987), and we used the number of strikes on each model as our index of female attractiveness.

## 3. Results

### 3.1. Effects of age on rates of water loss

WLR increased with age in both males and females ( $t$ -tests,  $P<0.001$  for females,  $P<10^{-4}$  for males; Fig. 1). This conclusion held when WLR was calculated on either a per individual or a mass-specific basis, indicating that changes in body mass (Fig. 2a) could not explain the increase

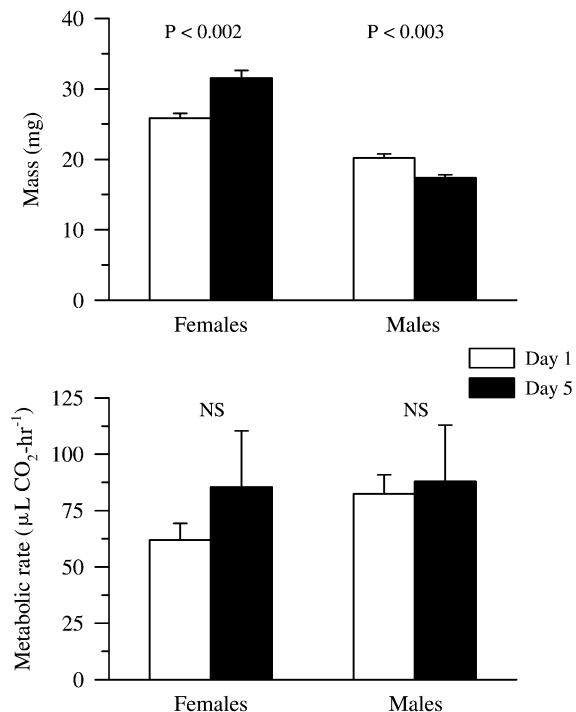


Fig. 2. Effects of age on body size and metabolic rates of houseflies. Means ( $\pm$ S.E.;  $n=6$ ).

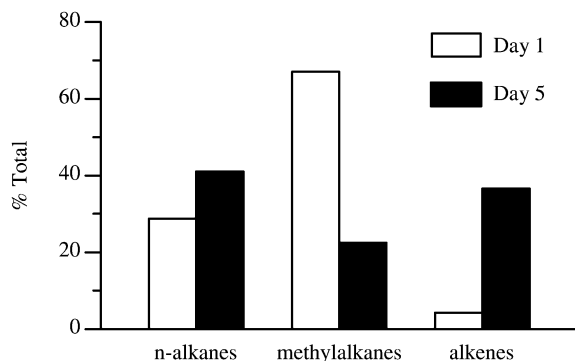


Fig. 3. Effects of age on cuticular hydrocarbon classes of female houseflies.

in water loss. Another possibility is that older flies were more active and, therefore, suffered greater respiratory losses through their spiracles. However, metabolic rates did not change as flies aged (Fig. 2b).

### 3.2. Effects of age on cuticular lipids

Gas chromatographic analyses provided similar age-related changes in lipid composition to those described previously (Nelson et al., 1981; Gibbs et al., 1995). Females accumulated ~10% (Z)-9-tricosene as they matured, as well as other alkenes (Fig. 3). The hydrocarbon composition of male flies also changed, with lower levels of methylalkanes and higher levels of *n*-alkanes in older flies (not shown).

Our FTIR results were similar to previous work (Gibbs et al., 1995);  $T_m$  values decreased with age in female, but not male flies (Fig. 4). Because WLR increased in both sexes (Fig. 1), these data suggest that water loss can increase in the absence of changes in lipid melting properties. Another factor to consider is lipid quantity, as older flies may have lower densities of surface lipids. Fig. 5 shows, however, that lipid quantities actually increased with age in both sexes, to a similar extent. Thus, higher rates of water loss were not caused by a decrease in the thickness of the cuticular lipid barrier.

### 3.3. Behavioral assays

We performed two series of mate-choice experiments using dead female models. In both sets of experiments, 5-day-old males were provided with

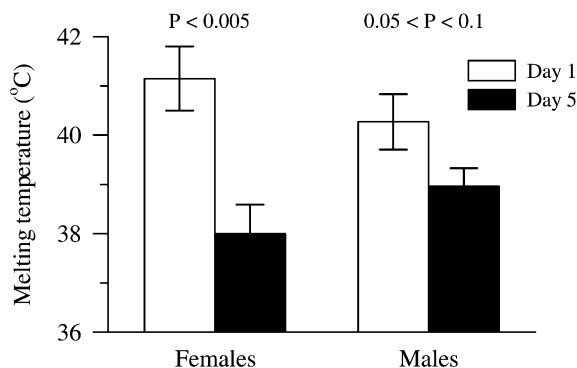


Fig. 4. Effects of age on melting temperatures ( $T_m$ ) of cuticular hydrocarbons. Means ( $\pm$ S.E.;  $n=6-7$ ).

a choice between models treated with either a high- $T_m$  pheromone mixture or a low- $T_m$  pheromone mixture.

#### 3.3.1. Experiment 1

The high- $T_m$  mixture in the first series contained 10% (Z)-9-tricosene and 90% *n*-pentacosane (w/w). This mixture melted at ~50 °C, as indicated by FTIR. The low- $T_m$  mixture contained 10% (Z)-9-tricosene and 90% *n*-hexadecane and melted at ~21 °C. Preliminary studies indicated that, in pure form, neither of the two alkanes had any apparent effects on male behavior. In particular, female models treated solely with *n*-alkanes elicited no mating strikes. Thus, males were given a choice of models containing a physiologically relevant concentration of pheromone, in either a solid or a melted hydrocarbon environment.

We staged a total of 60 choice tests lasting 20 min each. Some males were either inactive or

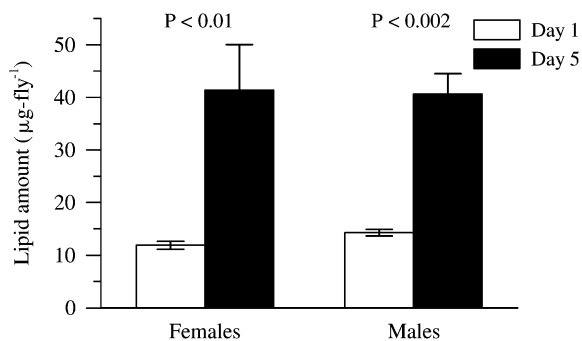


Fig. 5. Effects of age on quantities of cuticular hydrocarbons. Means ( $\pm$ S.E.;  $n=3-4$ ), *t*-tests performed on log-transformed data.

Table 1  
Results of heterogeneity  $G$ -test for behavioral experiment I: 10% tricosene + 90%  $n$ -hexadecane (low- $T_m$ ) or  $n$ -pentacosane (high- $T_m$ )

Factor	d.f.	SS	$P$
Pooled	1	0.167	>0.1
Heterogeneity	22	63.166	<0.001
Total	23	63.332	<0.001

Only males which performed at least five mating strikes were included in the analysis ( $n=24$ ). The pooled term is equivalent to a  $\chi^2$ -test and revealed no significant difference from a ratio of 1:1 in the number of mating strikes performed on low- and high- $T_m$  models.

spent most of their time trying to escape the arena, exhibiting little interest in either model. We therefore restricted our analyses to 24 trials in which the male attempted copulation with models at least five times. Males in these trials made a total of 384 mating strikes, 188 on the low- $T_m$  models and 196 on the high- $T_m$  models. The results of this experimental series are shown in Table 1 and were analyzed using a heterogeneity  $G$ -test (Sokal and Rohlf, 1995). The 'pooled' term in the  $G$ -test is equivalent to a  $\chi^2$ -test. Males exhibited no significant preference for either model treatment ( $P > 0.1$ ; Fig. 6). The significant 'heterogeneity' term in Table 1 indicates that some males exhibited a significant preference for one model over the other. Three males preferred the high- $T_m$  model, while two preferred the low- $T_m$  model.

### 3.3.2. Experiment II

Our second series of experiments used a similar design, except that the lipid mixtures contained 5% (2  $\mu$ g) methyl-branched alkanes, isolated from mature females. Previous work has implicated these compounds as modulators of male courtship behavior (Uebel et al., 1976; Rogoff et al., 1980; Adams and Holt, 1987; Adams et al., 1995). Again, the tricosene pheromone was present at 10%, with the remaining 85% consisting of  $n$ -pentacosane or  $n$ -hexadecane. The melting points of these mixtures were 22 and 50 °C, respectively. We restricted our analysis to 25 out of the 60 trials, in which males performed at least five mating strikes. Males made a total of 290 strikes, of which 111 were directed to the low- $T_m$  model and 179 to the high- $T_m$  model. This difference in the number of mating strikes on high- vs. low- $T_m$  models was highly significant ( $\chi^2$ ,  $P < 0.001$ ). A sign test comparing individual preferences revealed

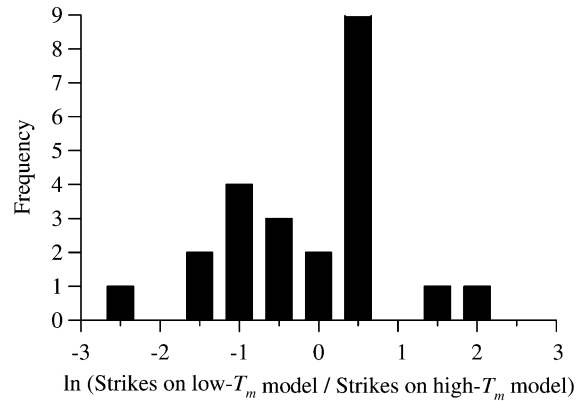


Fig. 6. Distribution of male preferences for pheromone-treated female models (Experiment 1). Mixtures included 10% ( $Z$ )-9-tricosene and 90%  $n$ -hexadecane (low- $T_m$ ) or  $n$ -pentacosane (high- $T_m$ ).

a highly significant bias towards the high- $T_m$  model ( $P < 0.01$ ; Fig. 7). A heterogeneity  $G$ -test indicated significant individual variation in model preference (Table 2). Seven males had a significant preference for the high- $T_m$  model, whereas only one male preferred the low- $T_m$  model. Note that these results contradicted our initial hypothesis of a preference for low- $T_m$  mixtures.

## 4. Discussion

The deposition of large quantities of unsaturated tricosene pheromone on the cuticle of the adult

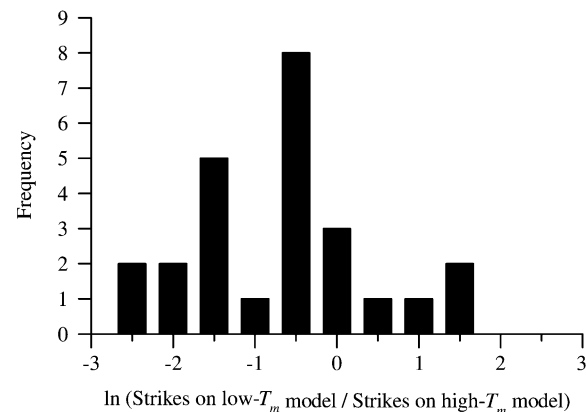


Fig. 7. Distribution of male preferences for pheromone-treated female models (Experiment 2). Mixtures included 10% ( $Z$ )-9-tricosene, 5% methylalkanes, and 85%  $n$ -hexadecane (low- $T_m$ ) or  $n$ -pentacosane (high- $T_m$ ). Note that the distribution of mating strikes is skewed towards high- $T_m$  lipid environments.

Table 2  
Results of heterogeneity  $G$ -test for behavioral experiment II: 10% tricosene + 5% methylalkanes + 85%  $n$ -hexadecane (low- $T_m$ ) or  $n$ -pentacosane (high- $T_m$ )

Factor	d.f.	SS	$P$
Pooled	1	16.09	<0.001
Heterogeneity	23	57.56	<0.001
Total	24	73.65	<0.001

Only males which performed at least five mating strikes were included in the analysis ( $n=25$ ). The pooled term indicates a significant departure from a ratio of 1:1 in the number of mating strikes performed on low- and high- $T_m$  models, while the significant heterogeneity term indicates individual variation in model preference. Post hoc tests revealed that seven males had a significant preference for the high- $T_m$  model, whereas only one male preferred the low- $T_m$  model ( $P<0.05$ ).

female housefly depresses the melting temperature of the cuticular lipids. This suggests a possible interaction between natural and sexual selection, with increased pheromone efficacy coming at the cost of increased evaporative water loss through the cuticle. However, we found no evidence of increased pheromone efficacy in a low- $T_m$  lipid environment, and we found increased WLR in flies of both sexes. As will be explained below, a working model for lipid heterogeneity across the cuticle may explain both results.

#### 4.1. Increased rates of water loss in mature flies

Flies of both sexes lost water more rapidly as they matured (Fig. 1), despite having three times the amount of surface lipid (Fig. 5). Water is lost from insects by three routes, any or all of which may be responsible for this increase: transpiration through the cuticle, diffusion through open spiracles, and excretion. Excretory water loss was easily detected as a large peak on the water loss recordings as fecal water evaporated. These events were observed for approximately half of the flies, but contributed less than 5% of total losses.

Respiratory water loss could also have increased, due to higher metabolic rates or loss of spiracular control. Flies of all ages were usually active in the respirometer, and in only a few cases was there any indication of discontinuous gas-exchange cycles (Lighton, 1994, 1996). These periods lasted only a few minutes, and carbon dioxide release never ceased completely, indicating that the spiracles were at least partially opened at all times. Thus, it was impossible to separate

spiracular from cuticular water loss in a rigorous manner. We estimated cuticular permeability by quantifying water loss during periods of minimal  $\text{CO}_2$  release. Our best estimate was that approximately three-fourths of water loss occurred via the cuticle, and that respiratory water loss contributed ~20% of the total. Because metabolic rates did not increase with age, respiratory losses do not appear to be the major factor causing the approximately twofold increase in total water loss.

This leaves changes in cuticular permeability as the most likely cause of increased WLR as flies aged. Similar conclusions have been reached using *Drosophila* (Nghiem et al., 2000; Gibbs and Markow, 2001). Melting points were  $>35^\circ\text{C}$ , so that the surface lipids would have been relatively solid at the measurement temperature for water loss ( $25^\circ\text{C}$ ). Some lipid melting could have occurred, however, especially in older females with lower  $T_m$  values. Partial melting can not explain increased water loss in males, however, since their  $T_m$  values did not change. Decreased lipid amounts could have increased cuticular permeability, but lipid quantities nearly tripled in both sexes. It should be noted that predicted correlations between water loss and lipid amounts or physical properties rest on the implicit assumptions that the surface lipids are evenly distributed across the cuticle and that their physical properties are also uniform. Several studies have demonstrated that insects may differentially deposit hydrocarbon types across the cuticle (McDaniel et al., 1984; Bagneres and Morgan, 1990; Young et al., 2000). This can cause lipid  $T_m$  values to vary by  $>30^\circ\text{C}$  within a single individual (Gibbs and Crowe, 1991; Young et al., 2000). Regional differences in lipid amounts and physical properties will affect cuticular permeability. The lipid compositions of both females and males change with age and as a result the lipid distribution and properties may be altered, with consequences for water loss. As will be discussed below, lipid properties may vary at finer spatial scales as well.

#### 4.2. Male preference for high- $T_m$ pheromone mixtures

Our initial hypothesis was that pheromones in a melted hydrocarbon environment would be more accessible to male chemoreceptors, due to their greater molecular mobility. In a solid lipid matrix, pheromone molecules may be blocked by other

lipids and, therefore, not detected. Our first series of experiments revealed no male preference, but the mixtures used contained an incomplete complement of pheromonal lipids. Although (*Z*)-9-tricosene is clearly the major compound used by males to identify females (Carlson et al., 1971), other lipids modulate male behaviors. In particular, methyl-branched alkanes increase male residence time on female models (Adams and Holt, 1987; Adams et al., 1995). This may increase the ability of males to discriminate between models.

Although our second set of experiments used relatively low levels of methylalkanes, their presence did indeed affect male behavior (Table 2). The total number of mating strikes was lower than in the first experimental series, which is consistent with males spending more time with the female models for each interaction. These experiments are not directly comparable, however, since they were performed at different times. The more important result is that, in contrast to our hypothesis, males preferred the high- $T_m$  models. An important consideration is that this hypothesis assumed that hydrocarbons on our models occurred in a homogeneous mixture.

In addition to the regional differences in lipid deposition noted earlier, surface lipids may vary at much finer scales. Cuticular lipids are mixtures of dozens to hundreds of compounds. Although the physical properties of pure hydrocarbons have been examined on several occasions (Blazyk and Rana, 1987; CRC Handbook, 1992; Gibbs and Pomonis, 1995), interactions between different hydrocarbons have received very little attention (Bonsor and Bloor, 1977; Small, 1986; Gibbs, 1995). Binary mixtures of alkanes appear to form homogeneous mixed crystals, as long as their chain lengths do not differ too much, with the melting point being a weighted average of the  $T_m$  values of the component HCs (Bonsor and Bloor, 1977).

Housefly lipids and our pheromonal mixtures contain significant quantities of unsaturated hydrocarbons, and the limited data available suggest that they exhibit complex interactions with saturated lipids. Mixtures of *n*-nonadecane with *n*-nonadecene have been examined using calorimetry, nuclear magnetic resonance, and X-ray diffraction (Small, 1986). Although these hydrocarbons are shorter than those typically found on houseflies and other insects, they are chemically similar and likely behave in a similar manner. Small (1986) concluded that alkenes and alkanes each form

distinct crystals at low temperatures. The alkene crystals melt at their  $T_m$  value, yielding a heterogeneous system containing both liquid alkenes and solid alkane phases. As the temperature increases, alkanes begin to melt into the alkene fraction, until the lipids form a completely melted mixture. Thus, over a broad temperature range these alkenes and alkanes do not form homogeneous mixtures. This inhomogeneity is not apparent when lipids are studied using FTIR (Gibbs, 1995).

#### 4.3. A phase separation model for cuticular waterproofing and pheromone efficacy in houseflies

We have previously proposed a working model (Gibbs, 2002) that extends the results of Small (1986) to cuticular lipids that contain alkenes, including the pheromone mixtures used in our behavioral experiments. Under this model, the low- $T_m$  mixtures would have consisted of homogeneous melted lipids, whereas the high- $T_m$  mixtures contained pure, solid pentacosane along with melted regions containing both tricosene and pentacosane. These 'lakes' would be enriched for (*Z*)-9-tricosene, which may have increased male responsiveness.

The phase separation model can explain our results in the second series of choice experiments, though not the lack of any differences in the first series. Methylalkanes present in the second experiment increase the time males spend at models (Adams and Holt, 1987; Adams et al., 1995), which may improve the ability of males to distinguish between models. It is also worth noting that their methyl groups would have disrupted the packing of *n*-alkane crystals, so that methylalkanes may have preferentially partitioned into the alkene fraction. If so, the melted lipids would have been enriched in all pheromone components. Thus, at least some chemosensory cells would have been exposed to potent pheromone mixtures.

In intact houseflies, our model predicts that the alkene fraction melts at  $\sim 0$  °C, reflecting the predominance of tricosene, with the alkane fraction progressively melting at higher temperatures up to  $\sim 50$  °C (Gibbs et al., 1995). Thus, liquid and solid phases will coexist at typical environmental temperatures, with all of the (*Z*)-9-tricosene occurring in the melted fraction. Phase separation also has implications for cuticular permeability: surface lipids may not provide a uniform barrier to water

loss, and attempts to link bulk lipid properties to water loss may be futile.

The phase separation hypothesis raises numerous questions, and further testing is certainly necessary (Gibbs, 2002). Other insects can deposit lipids with differing physical properties on different regions of the cuticle (Gibbs and Crowe, 1991; Young et al., 2000). Does *M. domestica* do this as well? If so, do these differences result in regional variation in cuticular permeability? How large are these regions, especially relative to the size of chemosensory cells? How applicable are biophysical measurements of simple lipid mixtures to lipid behavior in vivo? Phase separation has been described only for HC mixtures containing alkenes; how do non-HC lipids interact? These questions can only be addressed with new techniques, such as experimental methods that allow lipid properties to be measured on finer spatial scales than FTIR, and that allow in situ study. For example, atomic force microscopy allows investigation of the properties of lipids deposited on surfaces (Dufrene and Lee, 2000; Richter and Brisson, 2003), and polarized FTIR microscopy can assess the orientation and order of these molecules (Tamm and Tatulian, 1997; C. Park, personal communication). Theoretical and computational approaches will also be useful. Molecular modelling has been very successful in understanding lipid interactions in membrane bilayers (Ohvo-Rekila et al., 2002) and should be applicable to surface lipids.

Our results may have important implications for efforts to understand contact chemoreception. Even within *Musca*, contradictory results have been obtained (e.g. Rogoff et al., 1973; Uebel et al., 1976; La-France et al., 1989; Adams and Holt, 1987). We suggest that otherwise inert chemicals included in pheromone mixtures may modulate reception via indirect effects on lipid phase behavior. It is well established that the context in which signals are detected affects an animal's responses. For contact pheromones, an important aspect of context may be the physical state of the pheromone's milieu.

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