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Report

Social Context Influences Chemical Communication in *D. melanogaster* Males

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Summary

Chemical communication mediates social interactions in insects [1]. For the fruit fly, *D. melanogaster,* the chemical display is a key fitness trait because it leads to mating. An exchange of cues that resembles a dialogue between males and females is enacted by pheromones, chemical signals that pass between individual flies to alter physiology and behavior [2, 3]. Chemical signals also affect the timing of locomotor activity [4] and sleep [5]. We investigated genetic and environmental determinants of chemical communication. To evaluate the role of the social environment, we extracted a chemical blend from individual males selected from groups composed of one genotype and compared these extracts to those from groups of mixed genotypes. To evaluate the role of the physical environment, these comparisons were performed under a light-dark cycle or in constant darkness. Here, we show that chemical signaling is affected by the social environment, light-dark cycle, and genotype as well as the complex interplay of these variables. Gene-by-environment interactions produce highly significant effects on chemical signaling. We also examined individual responses within the groups. Strikingly, the response of one wild-type fly to another is modulated by the genotypic composition of his neighbors. Chemical signaling in *D. melanogaster* may be a "fickle" trait that depends on the individual's social background.

Results and Discussion

Cuticular hydrocarbons (CHs) form a film that separates an individual fly from its environment. Three structural classes of cuticular hydrocarbons are synthesized by *D. melanogaster* males: alkanes (straight chain hydrocarbons), alkenes (or monoenes, unsaturated hydrocarbons with one double bond), and methyl-branched alkanes (Figure 1A) [6]. We consistently find 23 distinct hydrocarbons belonging to these three chemical classes and differing in the length of their carbon backbone, plus a lone chemical called *cis*-vaccenyl acetate (cVA) on the outer surface of the fly (the cuticle). The precise functional role of these compounds is unknown for most of them. However, *D. melanogaster* male courtship behavior has been associated with a subset of cuticular hydrocarbons, most of them monoenes and cVA [7]. This underlies the hypothesis that hydrocarbons are the major sex pheromones of *D. melanogaster* [5]. In addition, insect hydrocarbons are thought to play a role in the regulation of water balance and to function as part of the immune system [6]. Based on their metabolism and diverse functions, it is likely that synthesis of these compounds is differentially regulated.

Complex traits such as the display of CHs are thought to be determined by genes, environmental influences, and, importantly, by the interaction between genes and the environment [8, 9]. Such gene-by-environment interactions are addressed implicitly by the molecular analysis of behaviors that rely on light-dark cycles, temperature [10], or food availability [11]. They are addressed explicitly by methods of quantitative genetics [8] that permit us to quantify the contribution of genes, environment, and their interactions to variation in cuticular hydrocarbon display [12–14].

We implemented the "host-visitor" paradigm (Figure 1B), an experimental design that manipulates the social and physical environment. In an earlier study, we applied this design to demonstrate that group composition influences circadian rhythms in locomotor activity and that this social influence is mediated by an unidentified airborne compound(s) acting as a pheromone [4]. Here, we recapitulate our earlier study to ask whether composition of the group affects the regulation of CHs and thus whether they might be involved in social communication. We extracted CHs from age-matched male flies maintained in homogeneous groups (40 wild-type or 40 mutants in each vial) or mixed groups (a mixture of 32 wild-type and 8 mutants in each vial). Comparing CH profiles from males of the same genotype but maintained in different groups permits us to analyze the contribution of the social group. Samples were collected hourly throughout the day in a light-dark cycle (LD) or in constant darkness (DD). We were therefore able to analyze effects of genotype, social group, lighting, and time of day (see Table S1 available online) on cuticular hydrocarbons in LD or in DD.

In this study, we focus on the male CHs and analyze sources of variability associated with their expression. This approach permits us to measure how much the social group contributes to chemical signaling in an individual. Three separate but related analyses are presented.

First, we use an analysis of variance (ANOVA) to quantify the variability in expression resulting from genotype and two other variables that make up the environment: social grouping and light. We looked for a statistical interaction between genotype and the environment because such interactions are often associated with complex traits [15]. We were particularly interested in the interaction between genotype and the social environment. The ANOVA was evaluated separately for each hydrocarbon. On average, the ANOVA model captures one-third of the total variability in the data. The ANOVA demonstrated that a main effect of genotype alone accounts for the largest variability in 17 of 24 compounds, and in particular for all monoenes. The social group alone accounts for the largest variability in the remainder of the compounds, especially for cVA and the methyl-branched alkanes. Light and time were less important (Table S1).

As noted above, a significant gene-by-environment interaction is expected for the ANOVA when applied to complex traits

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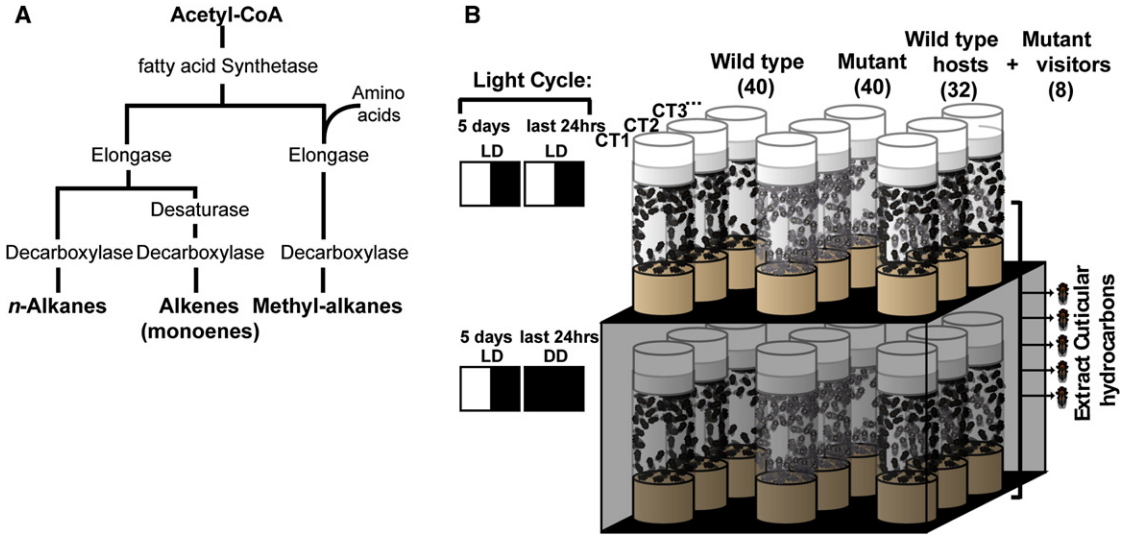

Figure 1. Overview of Cuticular Hydrocarbon Types and Synthesis Pathways, and Design of Group Effects Experiment

(A) Hydrocarbons destined for the cuticle are made in oenocytes from fatty acid precursors (Acetyl-CoA) by three related pathways. Simple lengthening of the fatty acids by elongase enzymes, followed by decarboxylation, produces saturated n-alkanes. Action of desaturase enzymes, followed by additional elongation and decarboxylation, produces alkenes that in males have only a single double bond and are called monoenes, the most abundant CH compounds. The third pathway begins with methylation, followed by elongation and decarboxylation, to produce methyl-alkanes. Each pathway produces compounds of varying lengths [6].

(B) Vials containing food and 40 male flies are maintained in a light-dark cycle for 5 days post eclosion. On the sixth day, vials are kept in either a 12 hr light/12 hr dark normal light cycle (LD) or in continuous darkness (DD). Control vials contain homogeneous groups of either 40 wild-type (Canton S) flies or 40 mutants (*y per^{0} w*). Heterogeneous group vials contain 32 wild-type ("host") and 8 mutant ("visitor") flies. 24 vials of each group and light-cycle combination are set up at start of experiment. At each of 24 hr on the sixth day, one vial per combination is removed and flies are sampled for hydrocarbon extraction [20, 30]. Contrasting hydrocarbon levels in LD versus DD vials reveal effects of light, and level changes between same genotype flies in homogeneous versus heterogeneous vials show "social" effects.

[9, 15]. We obtained a significant gene-by-environment interaction for all but one compound (5-C25:1; see Figure 2B and Table S1, columns GxS and GxL). The amount of variability explained by this interaction is different for each compound. The compound with the highest amount of variability explained by the interaction terms is 7-C23:1, a compound that plays an important role in male-male as well as male-female interactions [2, 16].

We were especially interested in how much of the overall variability is explained by the social group. Of the variability captured by the ANOVA, 43% involves the environment and at least 33% is explained by the social group variable and its interactions (Figure 2B; Table S1). The social group accounts for most (>50%) of this variability for five of these compounds (cVA, MeC24, MeC26, 7-C27:1, and C27). On average, the highest social effect is on the methyl-branched alkanes. Although the precise role of methylated CHs is not known, these compounds appear to be under sexual selection in natural populations, suggesting a role for this class of compounds as sex pheromones [17, 18]. We note that CHs can be transferred from one individual to another by contact [2], but the change in hydrocarbon profile that is evoked by the composition of the group in our assay cannot be explained by this mechanism (see Figures S1 and S2 for details). In summary, when viewed by ANOVA, a highly significant source of variability in our experiments comes from an interaction between genotype and the environment. Further, the social group exerts a highly significant effect on an individual's expression of CHs. These findings are consistent with the hypothesis that, like many behaviors, the expression of chemical signals is not strictly determined by genotype or the environment. Instead, it appears to be shaped by individual experience.

As a second approach, we use principle coordinate analysis, a method that accounts for variability in expression by placing hydrocarbons with similar patterns of variation together (PC1, PC2, etc.) (see Figures S1 and S2, Table S2) [19]. This method is similar to others commonly used in the analysis of hydrocarbon signals [18]. As a pattern detector, this principle coordinate analysis makes no assumptions about how the CHs should go together but, nevertheless, the observed patterns fit neatly onto the three chemical classes described above. Overall principle coordinate analysis captures 58% of the total variability in the data.

In theory, each hydrocarbon could show a different response to social group or light. However, in practice we have shown previously that features of chemical structure such as carbon chain length (PC1 axis) and chemical class (PC2 axis) account for 58% of the variability in wild-type males under these conditions [19], a higher fraction of the total variability than what we found in our first analysis with ANOVA described above. We find similar patterns of coexpression in the mutant strain (Figure S3). Such similarities between the two genotypes used here may be due to genetic or metabolic constraints that persist despite differences in timing and levels of expression for individual compounds.

We further applied an ANOVA to the PCs in an effort to understand whether genotype, social group, and light might influence different classes of compounds in different ways. PC2, which distinguishes monoenes from alkanes and methyl-branched alkanes, responds strongly to social group and genotype but much less to light (Figure 3A; Table S4). The position of a compound along PC2 is a highly significant predictor of the magnitude of social effects for each compound (Figure S4). This means that the expression of

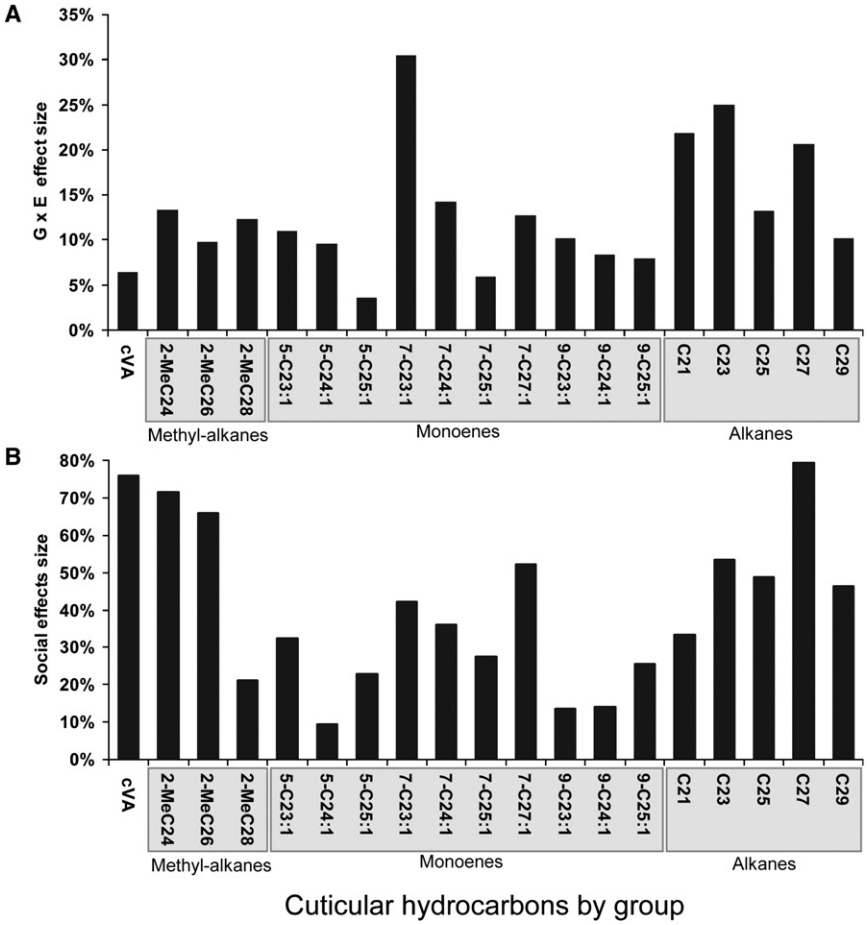

Figure 2. Social and Genotype by Environment Effects

Vertical axis: proportional effect sizes (η^2) of given effects as a percentage of size of all significant η^2. Horizontal axis: cuticular hydrocarbon compounds arranged by chemical group.

(A) Sum of all social effects (social, genotype × social, social × light cycle) as percent of total significant effects. The sum of all social effects is highly significant for each compound (Table S1).

(B) GxE as percent of total significant effects. GxE is the sum of GxL and GxS. All effects are significant (Table S1). Values shown from analysis of variance with factors for genotype, social, light, and time (see Supplemental Data).

monoenes, which include most known male courtship pheromones, is affected by the social group.

Being in a mixed group significantly (Figure 3A) reduces PC2 in constant darkness as well as light-dark cycles. This indicates that the social mix encountered by a male can stimulate a reduction in the proportion of monoenes to total CHs that he expresses. Indeed, presence in a mixed group causes a significant reduction in the proportion of total CHs because of monoenes (Figure S5; note also that total hydrocarbon level is higher in flies from mixed group, Figure S6). This reduction in monoenes is consistent with our observation that the expression of a desaturase gene critical to monoene synthesis is reduced in wild-type males from mixed compared to homogeneous groups [20].

Whereas monoenes correlate to PC2, carbon chain length is the chemical correlate of PC1 [19]. Like PC2, PC1 levels are strongly affected by group composition. However, whereas PC2 shows a strong response to the social group with little evidence of any interactions, PC1 reveals a strong interaction between the social group and light-dark cycle. This is revealed by the differences in the shapes of the curves in constant darkness versus a light-dark cycle for either group (Figure 3B; Table S1). Moreover, chain length is an excellent predictor of the light effect for each CH, with short chains showing much higher effects of light among monoenes and methyl-branched alkanes (Figure S8). Finally, PC1 reveals a strong interaction of social effect with time of day (Table S1). In the evening, levels of short chain compounds are higher in hosts than controls, whereas long chains are lower—during this time the two PCs predict 78% of the variation in social effect on different compounds (Figures S8 and S9, Table S5). This analysis shows that the social group tends to affect monoenes and methyl-branched alkanes that form strong clusters in PC2, whereas the social group together with light and time tends to influence hydrocarbons in PC1 that cluster by chain length.

The observation that PC1 and PC2 captures nearly 60% of the total variability in the regulation of CHs is remarkable. It suggests that social experience should have significant effects on this trait within only one genotype. We evaluated this possibility by comparing wild-type males reared in isolation (from the embryonic stage onward [21]) to wild-type males reared in groups. We predicted that social experience would affect the display of CHs for individuals of the same genotype. Indeed, we observed such responses. Whereas CHs on PC2 showed a consistent decrease for the monoenes among the isolates, PC1 showed an interaction between light and social treatment (see Figure S10, Table S6). Overall, these results are consistent with the previous experiment (Figure 3). They show that the CH profiles are influenced by social experience in this case (isolation or group rearing).

In summary, principle coordinate analysis demonstrates that social context influences the expression of pheromonal cues in *D. melanogaster* males. These effects tend to distinguish the classes of cuticular hydrocarbons: monoenes and methyl-branched alkanes display a strong response to the social group, PC2, and chain length predicts a strong interaction between social group and lighting regime, PC1.

The third approach emphasizes individual responses. Here we draw on work from the field of evolutionary theory. Moore and coworkers have proposed that social behavior defines an "interacting phenotype:" just as natural selection acts on individual traits that display more or less heritability (or plasticity for that matter), selection may also act on how individuals communicate and behave with one another [13, 22]. An interesting feature of this interacting phenotype is that each individual is a part of the other's environment and so individuals may contribute to the process of natural selection by their influence on others. This notion led to the theory of indirect genetic effects, an equation that relates the expression of one individual's phenotype to the strength of that individual's

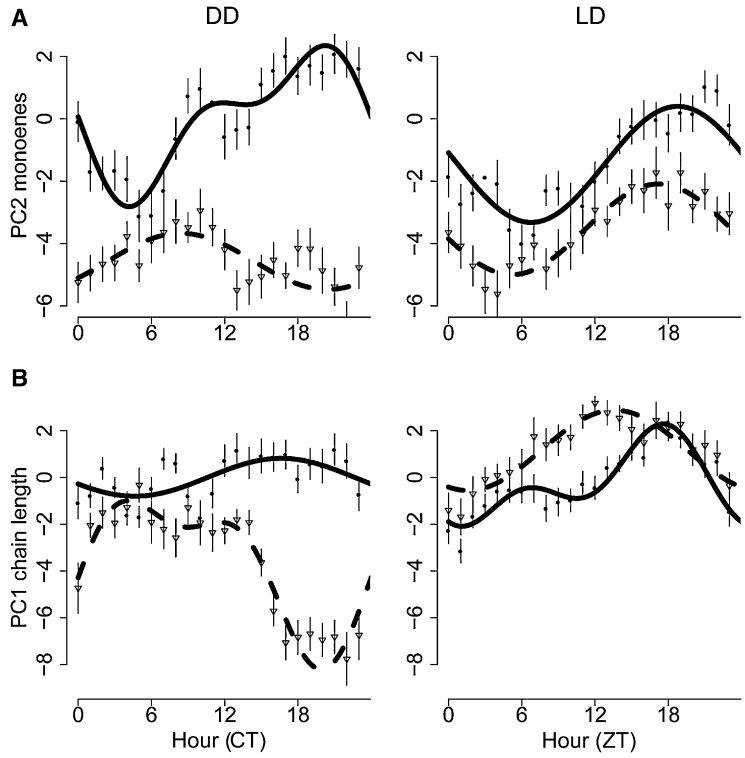

Figure 3. Group Composition Changes Principal Coordinate Levels and Time Course

Principle coordinate (PC) 1 and 2 are plotted here in two light conditions, with solid line for control wild-type males and dashed line for host males in mixed groups (see Figure 1 for experimental design). Group composition significantly affects average levels of each PC in each light condition (Table S4). (A) Average levels of PC2 plotted over time of day in DD and LD. Lower PC2 values mean reduced monoenes compared to other compounds, with social host flies lower at each time point in both DD and LD. (B) Average levels of PC1 plotted over time of day in DD and LD. Unlike PC2, the lighting regime interacts strongly with social group in PC1 levels, which contrast short to longer chain compounds. These differ significantly between social host flies and controls in DD and LD but in opposite directions and with a close to 180 degree phase shift from controls. Fourier curves are fit at significance level p = 0.05 (Supplemental Experimental Procedures). Points are 3 hr moving-average values ± SEM.

interactions with others of his or her species. Within the IGE equation, a coefficient (psi) represents the effect of others on an individual. If psi is 0 for some trait, then that trait is unaffected by other individuals; if psi is 1 or −1, then the trait is changed by the social environment to be similar to or different from others in the neighborhood; values of psi greater than or less than 0 provide a scale for evaluating the strength of genetic inheritance and that of social environment for a trait. The coefficient psi is a measure of an indirect genetic effect (IGE).

Such IGEs have been documented in mammals [23] and in flies [14, 24]; the phenomenon occurs between siblings as well as unrelated individuals. The demonstration of IGEs in wild-caught and laboratory-reared populations of *D. serrata* are of particular relevance here because the expression of cuticular hydrocarbons changed rapidly when males were exposed to females of a different genotype [14]. The quick response of these flies suggests that their CHs do not merely signify that a generic behavioral process has been released. Instead, the CHs define a specific response in real time to others in the group. We evaluated psi to assess the influence of the social environment on individual responses in CH signaling.

The host-visitor design (Figure 1) allows us to ask whether an individual male adjusts his profile of CHs according to other flies in his environment. Manipulating the genotypic composition of each group allows us to discriminate between genetic and social influences on the blend of cuticular hydrocarbons.

In our study, the IGE coefficient Ψ measures the magnitude of change in an individual's CHs because of his behavioral interactions with his neighbors in the vial. The influence of such "socializing" on an individual's CHs is given by the magnitude and sign of Ψ in three social environments: homozygous wild-type, homozygous mutant, and the mixed group. Within the mixed group, we call the wild-type individuals "hosts" and the mutants "visitors" in keeping with naming conventions from an earlier study [4]. The IGE model (see Supplemental Data, Equation E1) is a linear equation with Ψ coefficients for interactions in each context. We test three possibilities. The simplest null hypothesis is that Ψ is zero in all three contexts. This would indicate the absence of a social effect. If Ψ differs from zero, we may ask whether it is constant across all contexts. This second hypothesis would indicate the presence of social effects that are not linked to genotype and genotypic mix. The third hypothesis, when Ψ varies depending on the genotype of the interacting flies, would indicate the presence of a context-dependent IGE.

In the heterogeneous group, flies interact with males of their own genotype as well as with the other. We measured the strength of this within-genotype interaction in the mixed groups from coefficients $\Psi_{h,h}$ (the effect of hosts on hosts) and $\Psi_{v,v}$ (visitors on visitors) and in unmixed groups from $\Psi_{wt,wt}$ and $\Psi_{per0,per0}$ (Table S7). In homogeneous groups, Ψ is significantly greater than 0 for almost all compounds, rejecting the null hypothesis. This indicates that in homogeneous groups there is a strong positive social interaction effect between the group members. Further, we can reject the second hypothesis because Ψ is not constant across all contexts. Interestingly, in mixed groups the effect of visitors on hosts, $\Psi_{h,v}$, is positive for many compounds but many $\Psi_{h,h}$ and $\Psi_{v,v}$ values are not significantly different from 0; a few (for example, 5-C23:1) are significantly less than 0.

We have established an IGE that is dependent on social context. The correlation over compounds between $\Psi_{h,v}$ and $\Psi_{h,h}$ or $\Psi_{v,v}$ is significantly negative (Figure S11A). Although positive effects were observed in a homogeneous group (between flies of the same genotype), the mixed groups display positive effects that are largely due to interactions between flies of different genotypes. Moreover, in the mixed group the Ψ between genotypes is large when Ψ within genotypes is small.

Strikingly, the response of a first wild-type male to other wild-type males depends on the first male's social context (Figure 4A). Although the alkanes are unaffected by this response, monoenes and branched-methylated compounds are strongly related when wild-type males are grouped with other wild-type males; this similarity is significantly reduced when wild-type males are kept in a mixed group (Figures 4A and 4B). This effect of the social environment is especially pronounced among compounds that contain a double bond

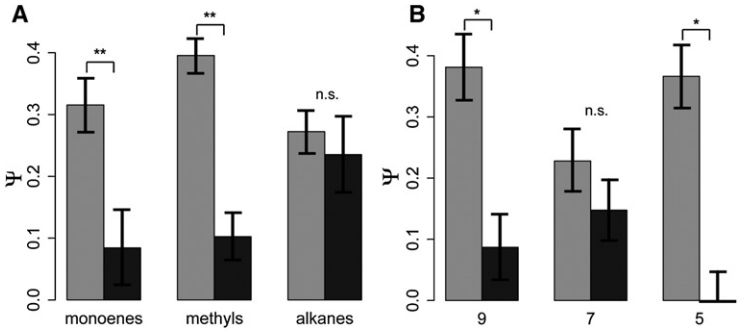

Figure 4. Indirect Genetic Effects of Wild-Type Flies Depends on Social Context

(A) Within-genotype interactions in unmixed and mixed groups. $\Psi_{w,w}$ (light gray bars, interaction of wild-type with other wild-type males in unmixed groups) compared to $\Psi_{h,h}$ (interaction of wild-type hosts with other wild-type hosts in mixed groups, dark bars), summarized by compound chemical type. In unmixed groups, within-genotype interactions are stronger than in mixed groups, for monoenes (paired two sided t test $t = 4.67$, $df = 9$, $p = 0.0012$) and methyl-branched alkanes ($t = 23.37$, $df = 2$, $p = 0.0018$), but not for alkanes ($t = 0.56$, $df = 7$, $p = 0.59$). Error bars are 1 SEM.

(B) Among monoenes, unmixed versus mixed group interaction difference depends on double bond position. 9-monoenes have greater indirect effects in unmixed groups (paired t test $t = 5.65$, $df = 2$, $p = 0.030$) as do 5-monoenes ($t = 7.40$, $df = 2$, $p = 0.018$), but the difference is not significant for 7-monoenes ($t = 1.62$, $df = 3$, $p = 0.203$).

associated with the 5th and 9th carbon in the hydrocarbon chain; however, we found no statistical difference associated with the 7th carbon, suggesting that the 5- and 9-monoenes are important for male-male social interactions (Figure 4B). We note that these data are consistent with the proposed role for 5-C23:1 as a signal that males use to suppress sexual behavior in other males [2]. The difference in pheromonal responses between males of the same genotype clearly depends more on their social environment than on their genotype.

We were able to link these IGEs to the analysis of variability in the data shown above. The structure of the CHs provides insight into the pattern of IGEs: the strong IGE effect of visitors on hosts is related to PC1, the first principal coordinate of hydrocarbon coexpression. For hosts and visitors in DD, there is a highly significant relationship between $\Psi_{h,v}$ and PC1 (Figure S11B). The effect of visitors on hosts corresponds to chain length; the effect is higher with shorter chain length and lower on compounds with long chain length. One interpretation is that our IGE analysis captures a rapid effect of interactions between flies. This short-term effect may occur over periods of hours or less in compounds with high responsiveness to light (the shorter chain compounds in PC1), whereas a more sustained response to group composition is detected in the monoenes (PC2).

We have studied sources of variability that affect the display of hydrocarbons on the fruit fly *D. melanogaster*. Clearly, the social environment influences this phenotype. The effects on Ψ (see Figure 4) suggest that monenes and methyl-branched compounds act as pheromones to convey social information. Further studies are required to establish rigorously whether these hydrocarbons are bona fide pheromones. Nevertheless, based on this demonstration that an individual *D. melanogaster* male adjusts its chemical display in response to the details of its social context, we can further study the fruit fly to understand the mechanistic basis of social relationships. Our data point toward mechanistic questions that can, in principle, be answered. For example, what cellular circuitry underlies the ability of a fly to know its group and shape its response to others? What molecular mechanisms underlie the synthesis of hydrocarbons (or other communication signals) and how are they regulated? How in response to others?

That these social interactions also occur in natural populations and in different species is supported by the observation that CHs have a social role in drosophilids from three continents (*D. melanogaster* [2, 25], *yakuba* [26], Africa; *D. paulistorum,* S. America [21]; *D. serrata*, Australia [14]). Pheromonal mechanisms that underlie important biological decisions like mate choice are not restricted to insects. They are also present in other animal groups including, fish, birds, reptiles, amphibians [27], and mammals [28, 29]. Ultimately, the genetic pathways that underlie pheromonal signaling may reveal the evolving basis of sociality.

Experimental Procedures

Details of fly culture and GC-FID hydrocarbon determination are as described [20, 30]. Hydrocarbon measurements were standardized against internal nC26 standards, then the FA transform [19] was applied to reduce the approximately lognormal data to a normal distribution. Clustering [31] and Fourier analysis are as described [19]. Statistical methods for IGE and partition of variance are described in Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, 13 figures, and 7 tables and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/18/1384/DC1/.

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