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Article

Social Experience Modifies Pheromone Expression and Mating Behavior in Male *Drosophila melanogaster*

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Summary

Background: The social life of animals depends on communication between individuals. Recent studies in *Drosophila melanogaster* demonstrate that various behaviors are influenced by social interactions. For example, courtship is a social interaction mediated by pheromonal signaling that occurs more frequently during certain times of the day than others. In adult flies, sex pheromones are synthesized in cells called oenocytes and displayed on the surface of the cuticle. Although the role of *Drosophila* pheromones in sexual behavior is well established, little is known about the timing of these signals or how their regulation is influenced by the presence of other flies.
Results: We report that oenocytes contain functional circadian clocks that appear to regulate the synthesis of pheromones by controlling the transcription of *desaturase1* (*desat1*), a gene required for production of male cuticular sex pheromones. Moreover, levels of these pheromones vary throughout the day in a pattern that depends on the clock genes and most likely also depends on the circadian control of *desat1* in the oenocytes. To assess group dynamics, we manipulated the genotypic composition of social groups (single versus mixed genotypes). This manipulation significantly affects clock gene transcription both in the head and oenocytes, and it also affects the pattern of pheromonal accumulation on the cuticle. Remarkably, we found that flies in mixed social groups mate more frequently than do their counterparts in uniform groups.
Conclusions: These results demonstrate that social context exerts a regulatory influence on the expression of chemical signals, while modulating sexual behavior in the fruit fly.

Introduction

Several recent studies of the fruit fly *Drosophila melanogaster* have shown that social context, defined as the size and genotypic composition of the social group, affects various behaviors including locomotor activity [1], aggregation [2], aggression [3], avoidance [4], feeding [5], reproductive behavior [6–9], and sleep [10]. Moreover, these studies emphasize an important role for chemical signals, particularly olfactory cues, in mediating these social effects.

Courtship and mating behaviors are perhaps the best known examples of social interactions involving the exchange and interpretation of various chemical signals in *D. melanogaster*. Some of these signals, like sex peptide [11] or cis-vaccenyl acetate [12–14], are transmitted along with sperm during copulation, whereas others, such as the cuticular hydrocarbons, are transmitted externally [7]. Several of these cuticular hydrocarbons are known to act as sex pheromones and modulate the probability of copulation. In *D. melanogaster*, sex pheromones are sexually dimorphic, i.e., males and females differentially express and respond to these cues. The *Drosophila* courtship ritual involves a set of pheromones for species recognition and sexual attraction that together define "sex appeal," as well as signals that inhibit male-male courtship [15, 16].

The circadian timing system influences social behavior [17]. The importance of circadian clocks for social interactions may be understood intuitively; in order to cooperate in a group activity, individual participants must know not only what to do, but also when to do it. In this way, the synchronization of behavior is necessary to organize the activity of the group. This influence of circadian rhythms is well documented in *Drosophila* where it temporally regulates the level of courtship and the probability of mating [8, 18–20].

The circadian timing system of adult *Drosophila* consists of specialized clock cells in various tissues [21, 22]. For example, certain clock cells in the brain (i.e., central clock cells) regulate the timing of locomotor activity, whereas antennal clock cells, residing outside of the central nervous system (i.e., peripheral clock cells) are thought to modulate responses to odorants, including social cues [23]. Peripheral clock cells have also been shown to exist in many other tissues including the eyes, wings, forelegs, guts, Malpighian tubules, and testes, but the function of clock cells in these tissues remains unclear [21, 22]. To date, little is known about the influence of circadian clocks on the production of chemical signals that define social responses, such as the sex pheromones. Based on the circadian nature of courtship behavior, we hypothesized that oenocytes, the pheromone-producing cells, contain functional peripheral clocks that regulate pheromone synthesis.

Here, we report that the expression of sex pheromones is clock regulated. We provide evidence that a circadian clock mechanism is present in the oenocytes and that this oenocyte clock regulates the production and emission of sex pheromones. In addition, manipulating the genotypic composition of social groups affects the circadian clock mechanism such that genotypic heterogeneity within the group reduces the amplitude and mean accumulation of clock gene transcripts in the head and in the oenocytes and alters the display of sex pheromones on the cuticle. Consistent with the modulation of pheromone productions, social interactions also alter the temporal profile of mating behavior, resulting in an increase in the daily total number of matings performed by wild-type males. The data demonstrate that the social environment can modify the endogenous clock mechanism governing sex-pheromone synthesis and influence sexual behavior in *Drosophila melanogaster*.

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Oenocytes Contain a Peripheral Clock

In insects, a specialized cell type referred to as oenocytes (Figure 1) is thought to be required for the production of cuticular hydrocarbons [24–28]. In *D. melanogaster,* feminization of male oenocytes results in the expression of female hydrocarbons on the male body surface [26]. These studies and others have demonstrated that cuticular hydrocarbons represent the output of the oenocytes [29].

The adult *Drosophila* oenocytes are located in the abdomen and are organized in metameric stripes extending along the inner cuticular surface of each tergite from the dorsal midline, adjacent to the dorsal vessel, laterally to the spiracles (Figures 1A–1E). These dorsal oenocyte clusters are composed of tightly grouped mononucleated cells, which form ribbon-like monolayers (Figure 1B and inset). Other, more ventral, oenocyte clusters exist on the inner surface of the abdominal sternites (Figure 1A) [30]. A basal lamina ensheaths the oenocytes, as has been described for ectoderm-derived tissues in other insects, and may account for the compact appearance of the monolayer (Figures 1D and 1E) [31]. When viewed in cross section, the interface between the oenocytes and the cuticle becomes apparent (Figures 1F and 1G). Positioned at the posterior edge of each segment, the oenocytes lay near to the flexible intersegmental membrane joining adjacent cuticle plates (Figures 1F and 1G). Although the mechanism by which hydrocarbon compounds are deposited onto the surface of the cuticle is not known, the close association of the oenocytes with the cuticle and intersegmental membrane suggests a route for their release to the outer body surface.

To determine whether oenocytes are circadian clock cells, we investigated whether these cells display features of the molecular clock mechanism defined by the cyclical expression of the core clock genes, *period* (*per*), *timeless* (*tim*), and *Clock* (*Clk*) [21]. In archetypal clock cells, the temporal profiles of *per* and *tim* RNA accumulation peak during the early night, whereas *Clk* peaks at an approximately opposite time in the daily cycle. This temporal pattern of clock gene expression defines the molecular signature of the circadian clock for both central and peripheral clock cells. The expression of these core clock genes are expected to be abnormal in the arrhythmic per^0 mutant. Consistent with these features of the clock, all three core clock genes are expressed in oenocytes and their temporal patterns of wild-type expression are significantly sinusoidal ($p \leq 0.0001$), whether in constant darkness (DD) or in a light-dark cycle (LD) (Figures 2A and 2B; Figure S2 and Table S1 available online). In addition, the phase relationships between *per*, *tim*, and *Clk* are consistent with the canonical *Drosophila* wild-type circadian clock mechanism in DD and LD [21, 22]. The peak in *per* RNA occurs about 1.5 hr earlier than *tim*, whereas the peak in *Clk* RNA occurs about 12 hr before *per* (Figures 2A and 2B; Table S2). Similarly, per^0 oenocytes display a disruption of the temporal pattern of expression in DD (Figure 2C; Tables S1 and S3). Together, these findings indicate that the oenocytes are peripheral clock cells.

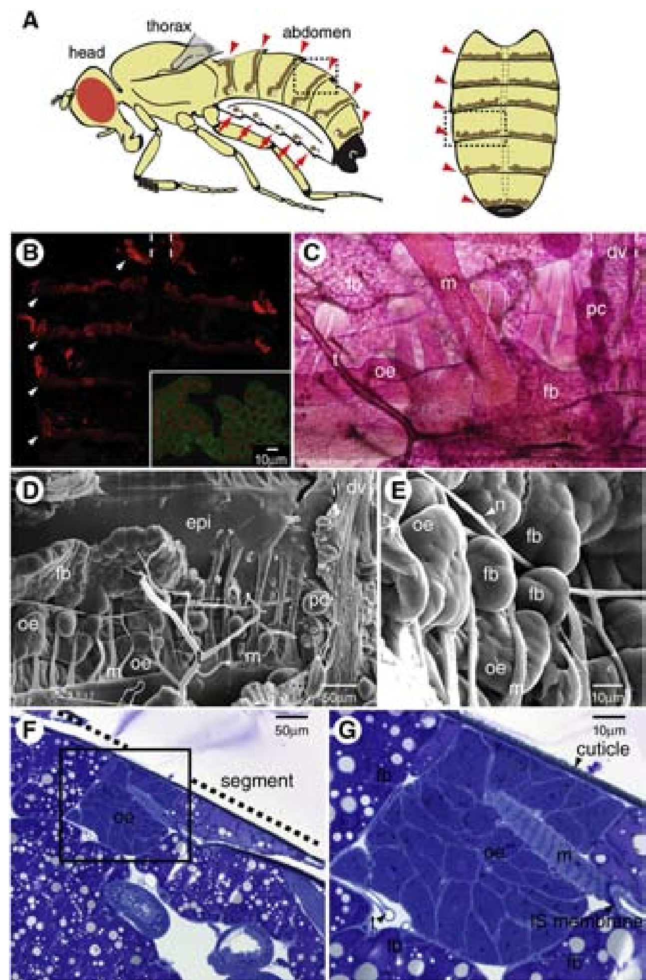

Figure 1. Cytology of Adult *Drosophila* Oenocytes

(A) Cartoons showing the location of the oenocytes in the abdomen of an adult fly. The profile of a fly is shown on the left. The dorsal aspect of a fly abdomen is shown on the right. The oenocytes lie in the posterior region of each abdominal segment, immediately beneath the surface of the cuticle. Dashed box in the left image frames the position of the sagittal section shown in (F) and (G). Dashed box in the right panel frames the location of the images shown in (B)–(E). Oenocytes, dark brown; dorsal oenocyte clusters, red arrowhead; ventral oenocyte clusters, red arrow.

(B–E) Filet preparations of adult male abdomens.

(B) Individual oenocytes are marked by a nuclear form of β-gal (UAS-NZ.lacZ; red) and a cell-membrane-targeted form of GFP (UAS-mCD8::GFP; inset only, green), each misexpressed via the Gal4 driver, 1407-Gal4 (Figure S1). Abdominal segments A1 to A5 are shown. Arrowheads demarcate the posterior edge of each segment. The dorsal vessel is marked by dashed lines.

(C) Hematoxylin and eosin staining of a single abdomen hemisegment. Oenocytes are strongly eosinophilic and appear as a dark pink band of cells extending along the posterior edge of the segment. A4 hemisegment is shown.

(D and E) Scanning electron microscope images of a single abdominal hemisegment.

(E) Increased magnification of oenocytes shown in (D).

(F and G) Sagittal section of an adult male abdomen stained with toluidine blue.

(G) Increased magnification of oenocytes shown in (F). Dotted lines bound the anterior and posterior aspects of the dorsal vessel located at the midline. dv, dorsal vessel; epi, epidermis; fb, fat body; m, muscle; n, nerve; pc, pericardial cell; oe, oenocytes; t, trachea; IS membrane, intersegmental membrane.

A Peripheral Clock Regulates Cuticular Pheromones

Because oenocytes have functional molecular clocks, we hypothesized that the expression profile of cuticular pheromones may display circadian rhythmicity. Of the 23 hydrocarbon compounds found on the surface of the male cuticle, 4 are thought to act as male sex pheromones: 5-tricosene or 5-C23:1 (5-T), 7-tricosene or 7-C23:1 (7-T), 7-pentacosene or 7-C25:1 (7-P), and 9-pentacosene or 9-C25:1 (9-P) (Figure 3A) [7]. We observed that these compounds cycle with a circadian rhythmicity. Although not all are identical in pattern, wild-type levels of these compounds in DD are generally lower at times during the subjective day (times 0–11) than

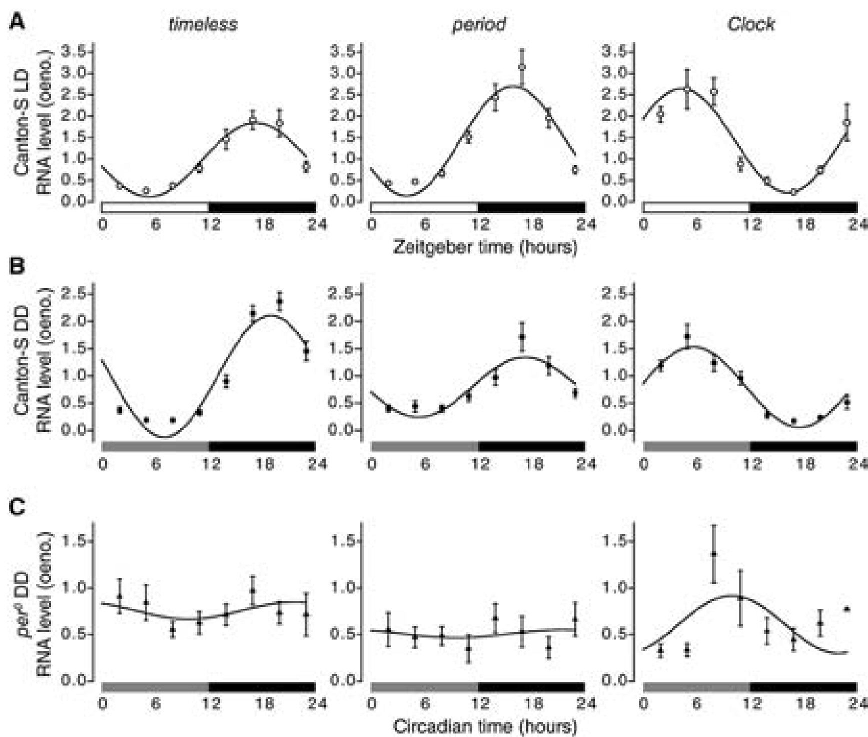

Figure 2. Oenocytes Contain a *per*-Dependent Clock

The temporal patterns of *tim*, *per*, and *Clk* RNA expression in wild-type and per^0 oenocytes as determined by quantitative RT-PCR. These patterns indicate a functional clock in the oenocytes for the wild-type in LD (A) and DD (B). The functional pattern is disrupted for per^0 in DD (C). Total RNA was isolated from dissected oenocyte preparations. Best-fit cosine curves (solid lines) are fitted to RNA expression values ± SEM (Tables S1–S3). Each time point represents the average of three replicate qPCR reactions from three independent experiments (n = 9). WT, wild-type; DD, first day constant darkness; WT LD, open circles; WT DD, filled circles; per^0 DD, filled triangles.

during the subjective night (times 12–23) (Figure 3B). Furthermore, the pheromone quantities on wild-type males compared with per^0 males differed at most times throughout the day (Figure 3B).

Hour-by-hour temporal patterns of hydrocarbon accumulation of wild-type and per^0 males were determined to evaluate whether the two genotypes displayed similar patterns of expression, albeit at different levels. Similarity between temporal patterns was assessed by evaluating the strength of the correlation between per^0 and wild-type temporal profiles in DD. No significant correlation was evident in DD for three of the four compounds: 5-T, 7-T, 7-P (9-P was correlated between the two male genotypes p = 0.0014). The daily temporal pattern of accumulation for these three courtship cues is thus *per* dependent in DD and at least partially dependent on a circadian clock.

Although constant darkness is used to assess whether an endogenous mechanism drives daily rhythms in the absence of environmental timing cues, we also evaluated the effect of such cues by comparing the wild-type levels of the four pheromones in DD to those in a more natural LD cycle (Figure 3C). Significant differences between DD and LD in compounds 7-P and 9-P are evident only around the transition from day to night, whereas compounds 5-T and 7-T are significantly elevated in DD compared to LD during subjective midday as well as during the subjective night. Further, correlation analysis of wild-type temporal patterns in LD and DD reveal a strong association for 5-T (r = 0.671, p < 0.001), 7-P (r = 0.756, p < 0.001), and 9-P (r = 0.689, p < 0.001), but not 7-T (r = 0.419, p = 0.05). A striking reduction in amplitude of the daily pattern is evident in LD when compared to DD for 5-T (p < 0.003) and 7-T (p < 0.05), although no difference is apparent for 7-P (p = 0.63) and 9-P (p < 0.13) (Figure 3C). In summary, male courtship pheromones accumulate on the body surface in a *per*-dependent pattern in DD (Figure 3B), and this pattern is light sensitive (Figure 3C).

We considered the possibility that the *per*-dependent pattern of hydrocarbon accumulation might be influenced by the central clock and may represent an indirect consequence of behavioral rhythmicity. For example, rhythmicity in feeding behavior could influence the temporal availability of metabolites required for hydrocarbon production. The transgenic *per7.2:2* strain was employed to determine whether the temporal profile of hydrocarbon accumulation requires a peripheral clock [32]. In this strain, rhythmic expression of PER protein is restricted to a subset of clock neurons in the brain essential for generating locomotor behavior rhythms; in peripheral tissues, PER is completely absent [33]. In the *per7.2:2* strain, the oenocyte clock is arrhythmic (Figure S3A). If the display of cuticular hydrocarbon pheromones is driven by PER-positive neurons in the brain or by behavioral rhythmicity, then the temporal pattern in the *per7.2:2* strain, even in the absence of a functioning oenocyte clock, should be the same as that of wild-type control. In constant darkness, the wild-type control and *per7.2:2* males displayed significantly different amounts of 5-T, 7-T, 7-P, and 9-P at all time points (Figure S3B). Moreover, no significant correlation was evident between *per7.2:2* and wild-type in the temporal patterns of these compounds. These observations strengthen the hypothesis that cuticular hydrocarbon accumulation is regulated by a *per*-dependent peripheral clock mechanism in the oenocytes and is not driven or significantly affected by central clock cell functions.

desat1 Is a Clock-Controlled Gene in the Oenocytes

We next investigated a potential mechanism by which the oenocyte clock may regulate pheromone expression patterns. The metabolic pathways for hydrocarbon synthesis yield three chemical classes: straight chain saturated compounds, unsaturated compounds, and methyl-branched compounds. All of the pheromones studied here are monounsaturated hydrocarbon compounds (Figure 3A). We assessed the oenocyte expression of *desaturase1* (*desat1*), a gene that encodes a Desaturase enzyme [34, 35]. DESAT1 activity directly affects only those compounds with double bonds at the 7- position, e.g., 7-T and 7-P [34]. However, consistent with previous reports [36], we have observed that a mutation affecting *desat1* broadly alters the expression of all unsaturated compounds as well as other classes of compounds. Manipulation of *desat1* can alter

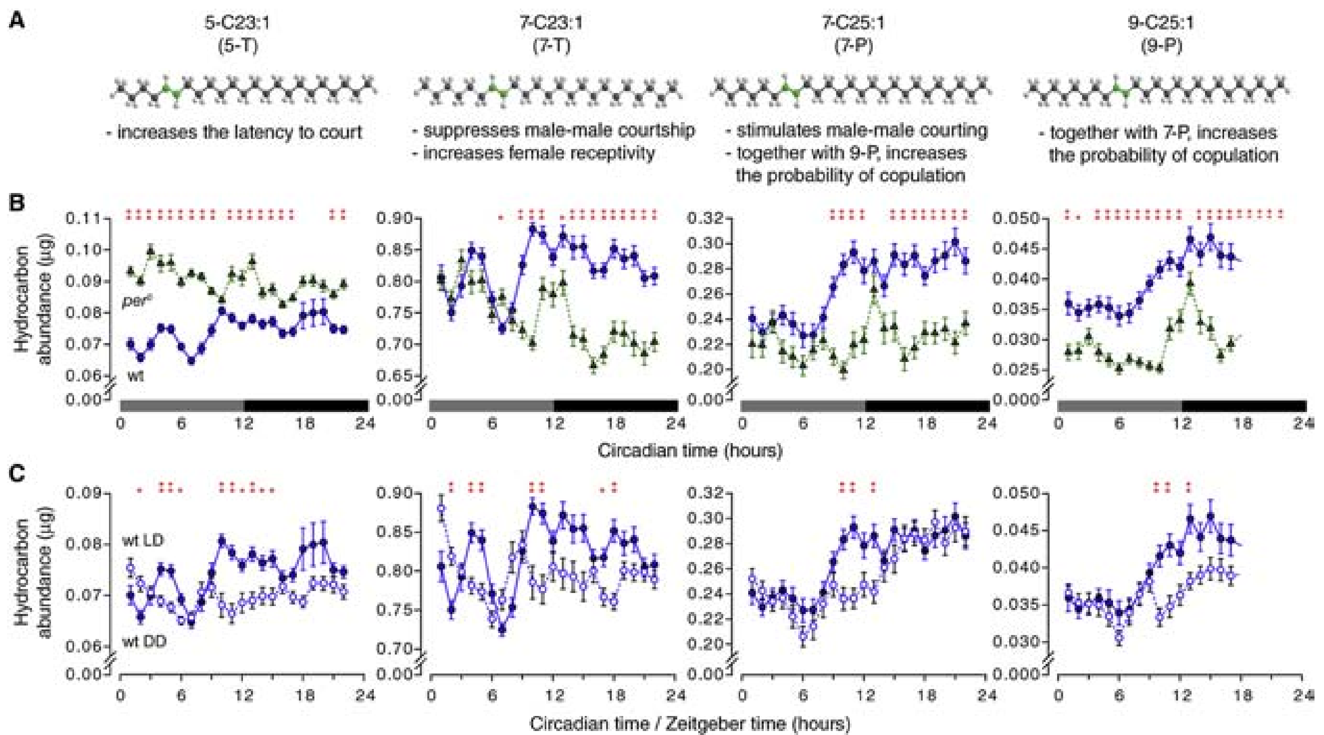

Figure 3. Cuticular Hydrocarbon Accumulation Is Regulated by a *per*-Dependent Clock and Is Influenced by Exposure to Light

(A) Structural representations of hydrocarbon compounds 5-T, 7-T, 7-P, and 9-P. Each consist of a carbon chain (23C or 25C) with a single double-bond at position 5, 7, or 9 (green).

(B and C) Comparisons in the temporal profile of the indicated hydrocarbon compound between wild-type and per^0 males in DD (B) and between wild-type males in DD and in LD (C). Shown are values calculated from a 3 hr moving average ± SEM (n = 27 to 45 per time point). Asterisks (red) indicate significant differences between genotypes or treatments as determined by a two-sided Student's t test (**p < 0.01; *p < 0.05; data corrected with Benjamini-Hochberg False Discovery Rate q = 0.1). WT, wild-type; WT DD, filled blue circles; WT LD, open blue circles; per^0 DD, filled green triangles.

both the blend of cuticular pheromones and male courtship behavior in *Drosophila* [7, 36]. We predicted that *desat1* would be expressed in the oenocytes and regulated by the oenocyte clock, and thereby may control the cyclic expression of cuticular hydrocarbons.

Consistent with these hypotheses, we observed that *desat1* is expressed in oenocytes and a sinusoidal expression pattern is evident under both DD and LD conditions ($p < 0.0001$) (Figure 4A). Like *Clk*, the temporal profile of *desat1* mRNA peaks during the subjective day with a trough at night. A fixed phase relationship occurs between *Clk* and *desat1* in oenocytes; on average, peak expression of *desat1* occurs approximately 4 hr after the peak in *Clk* expression (p = 0.023; Table S2). In addition, the circadian pattern of *desat1* expression is disrupted in per^0 flies in DD (Figure 4A). In these arrhythmic mutant flies, the *desat1* expression pattern is not significantly different from a flat line.

Although the above results demonstrate that *desat1* is expressed in oenocytes and may be clock regulated, the peak-to-trough variation in *desat1* expression, though statistically significant, is less than 2-fold in DD and in LD (Figure 4A). Similar results were obtained when we examined the temporal patterns of DESAT1 protein extracted from oenocytes at different times in the circadian cycle (Figure S4). Despite the small change, we hypothesized that circadian regulation of *desat1* expression is relevant to the daily pattern of pheromone accumulation. To further investigate this, we obtained a *desat1* hypomorphic mutant stock, $desat1^{1573\text{-}1}$, containing a *P*-element insertion in the *desat1* locus, and a control stock, $desat1^{1573\text{-}N2}$, formed by a precise excision of the insert [36]. Comparison of transcript, protein, and hydrocarbon levels in flies homozygous or heterozygous for the insertion and homozygous for the excision showed a monotonic relationship between levels of *desat1* transcript, protein, and the accumulation of both 7-P and 7-T (Figures 4B–4D; Figure S5). The degree of the difference seen among these flies reveals that a 50% change (i.e., less than a fold change) in transcript yields detectable changes in hydrocarbon accumulation.

The above observations support the interpretation that *desat1* is under clock control in the oenocytes and that the temporal lability of *desat1* transcript and protein affects the levels of pheromonal expression. It is clear that the observed relationship between *desat1* transcript, protein, and hydrocarbon expression encompasses the variation of wild-type values observed within a day (Figure 4D; Figure S5). This relationship between relatively subtle changes in *desat1* mRNA and the consequent effects on 7-P and 7-T accumulation provides suggestive evidence that low-amplitude rhythms in *desat1* RNA are functionally relevant.

Social Context Affects Clock Gene Expression and Pheromone Output

Pheromonal communications influence the physiology and behavior of interacting individuals within a group. As such they play an important role in the organization of social interactions [37]. However, social interactions are dynamic. Next, we evaluated whether social experience affects the temporal control of pheromone accumulation by comparing circadian clock

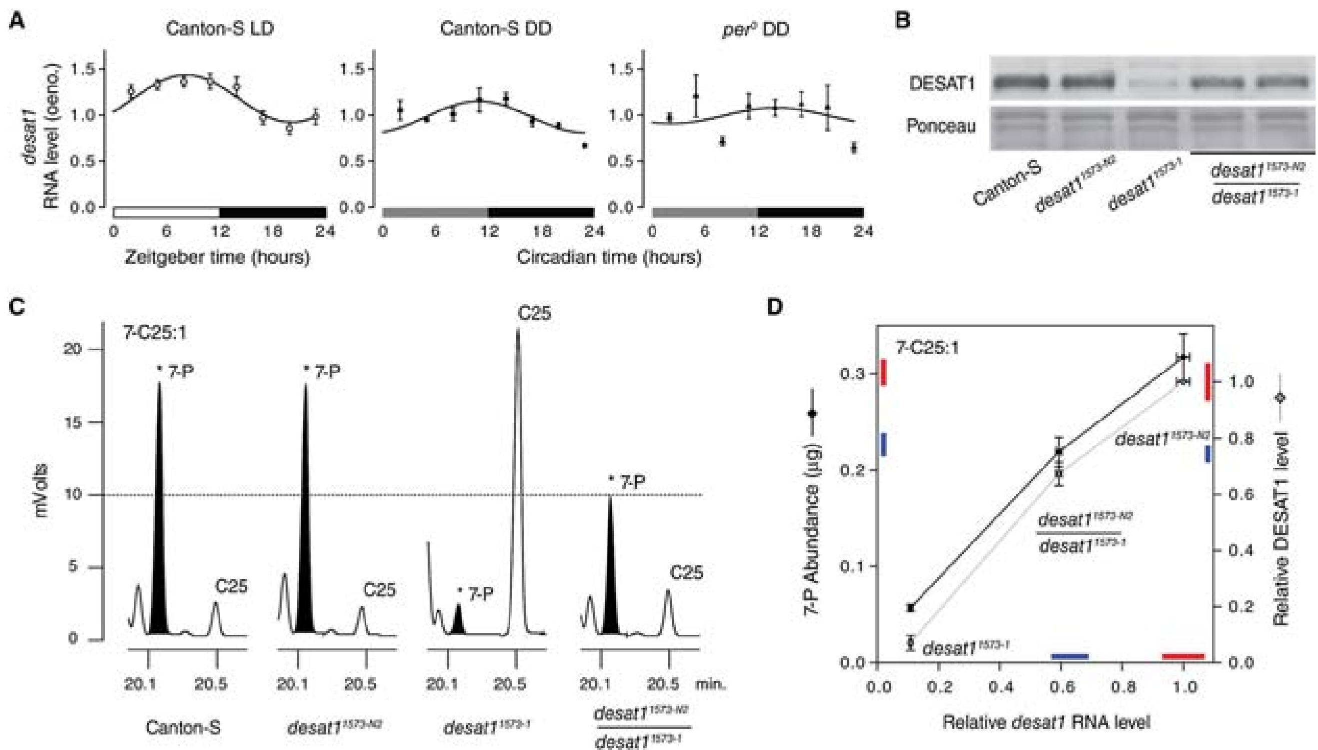

Figure 4. Cuticular Hydrocarbon Accumulation Is Regulated by the Expression Level of *desat1*

(A) The temporal patterns of *desat1* RNA expression in wild-type and per^0 oenocytes as determined by quantitative RT-PCR. Genotype and lighting conditions as indicated. Best-fit cosine curves (solid lines) are fitted to RNA expression values ± SEM (Tables S1–S3). RNA samples were the same as that used in Figure 2. Each time point represents the average of three replicate qPCR reactions from three independent experiments (n = 9), except for wild-type *desat1* expression in DD (n = 6). WT, wild-type; DD, first day constant darkness; WT LD, open circles; WT DD, filled circles; and per^0 DD, filled triangles.

(B) Western blot analysis of oenocyte extracts isolated from the indicated genotypes and labeled with an DESAT1 antibody. $desat1^{1573\text{-}N2}$ and $desat1^{1573\text{-}1}$ refer to control and homozygous *desat1* mutant genotypes, respectively. $desat1^{1573\text{-}N2}$ / $desat1^{1573\text{-}1}$ refers to the heterozygous *desat1* mutant genotype. Ponceau staining of the blot served as the loading control.

(C) Representative gas chromatographs displaying the amounts of 7-P (asterisks) and C25. Genotypes as in (B). The area under each peak corresponds to amount of each compound. The output of the flame ionization detector (FID) displaying the height of each peak (mVolts) and the corresponding retention times (min) is shown.

(D) Standard curve relating the level of *desat1* RNA to the level of 7-P (black line) and Desat1 (gray line) in genotypes shown in (B) and (C). The plotted lines may be compared to daily high and low values observed for *desat1* RNA (see [A]; LD), 7-P (see Figure 3C; WT LD), and Desat1 protein (see Figure S4; LD) as indicated by red and blue bars, respectively, positioned next to the indicated axis (bar length corresponds to ±SEM).

gene expression in the heads and oenocytes of wild-type males from homogeneous social groups with that of wild-type males from genotypically mixed groups (containing both wild-type and per^0 flies). In these tests, homogenous social groups consisted of 40 genotypically similar individuals per vial per time point. Heterogeneous groups consisted of a majority of 32 wild-type males, called "hosts," housed with a minority of 8 per^0 males, the "visitors." This 32:8 host:visitor ratio has been previously shown to maximize the effects of heterogeneous groups in this assay [1].

The overall temporal pattern of expression was similar in oenocytes and heads for both hosts and controls (Figures 5A and 5B; Tables S4 and S5). Interestingly, however, the social manipulation led to pronounced differences in the level and amplitude of gene expression. The amplitude of *tim* and *Clk* profiles, as well as the relative level of expression for *per*, *tim*, and *Clk*, were significantly reduced in the oenocytes of hosts compared to wild-type controls from homogeneous groups (Figure 5A; Tables S4 and S6). Like the clock genes, *desat1* expression was also reduced in host oenocytes relative to controls (Figure 5A; Tables S4 and S6). In heads, *per* and *Clk* expression were reduced in hosts compared to controls at each time point, as well as in the overall mean (taken across the day). The shape and amplitude of the respective expression curves, however, did not change in heads (Figure 5B; Tables S4 and S6). No significant difference was detected in the levels or pattern of *tim* expression. Thus, social context alters the patterns of clock gene expression within the oenocytes and heads, as well as the temporal relationship between the two tissues (Table S7). The mechanism by which social interactions produce this modulation of the molecular processes underlying such circadian timing systems remains to be elucidated.

The effects of social context on *Clk* and *desat1* expression in male oenocytes led to the prediction that patterns of cuticular hydrocarbons would also be altered by social context. To test this hypothesis, we compared sex-pheromone levels males from mixed versus homogeneous groups in DD (Figure 5C) and LD (Figure S6A). In hosts, the levels of 5-T and 7-T were significantly elevated over that of the controls during the second half of the subjective night and the latter parts of the subjective day in DD. Host levels of pheromones 7-P and 9-P, however, differed from controls mainly around the transition from subjective day to night (Figure 5C). The overall patterns of accumulation were not significantly correlated, indicating differences in expression patterns between hosts and controls

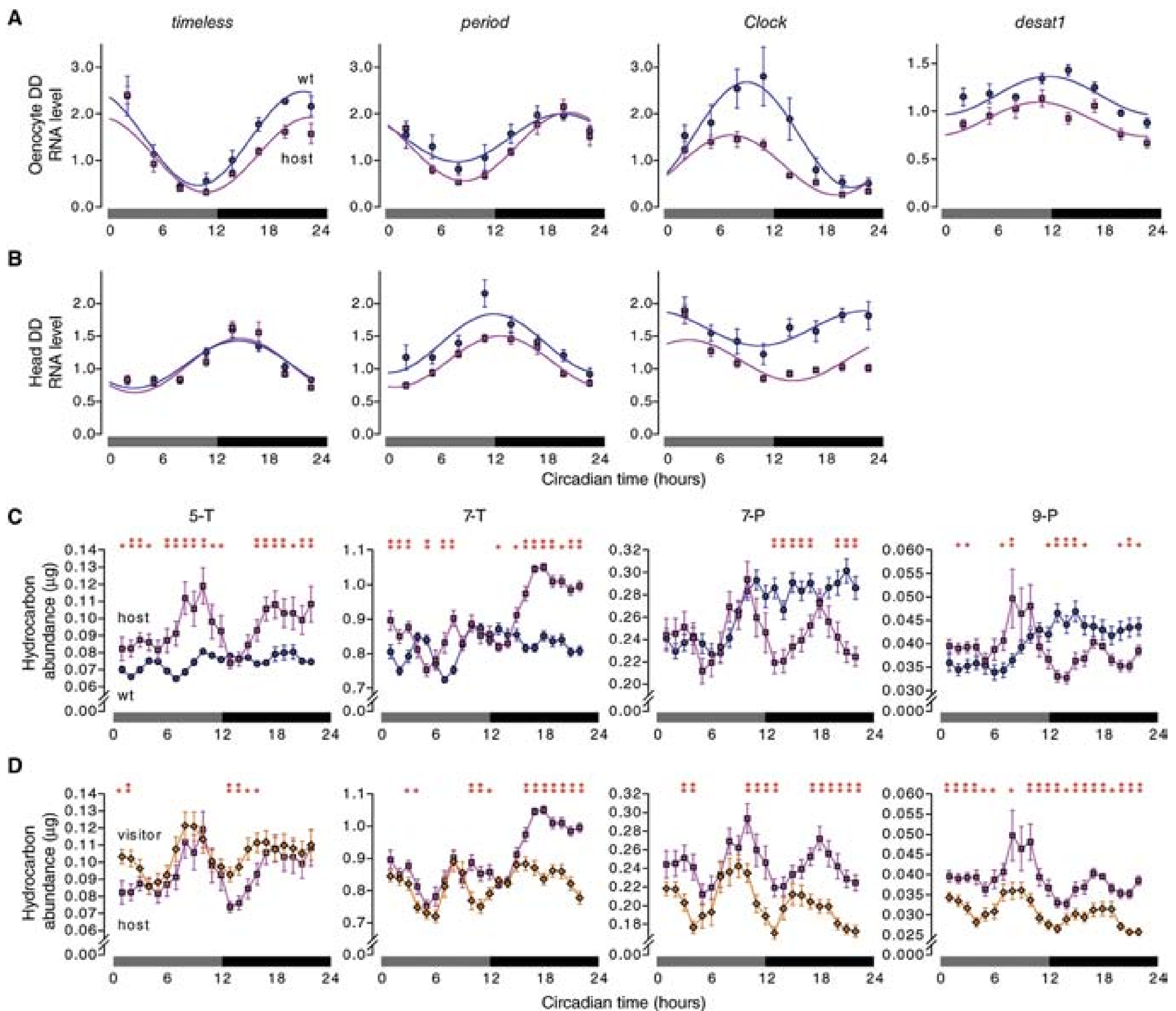

Figure 5. Social Interactions Affect the Temporal Profile of Gene Expression and Cuticular Hydrocarbon Accumulation

(A and B) The temporal patterns of *tim*, *per*, *Clk*, and *desat1* expression in wild-type control and host oenocyte preparations (A) and whole-head extracts (B) as determined by quantitative RT-PCR analysis. Oenocytes and heads were isolated from the same flies. Best-fit cosine curves (WT, wild-type; WT, blue line; host, magenta line) are fitted to RNA expression values ± SEM (WT DD, blue circles; host DD, magenta squares). Expression was assayed on the second day of constant darkness. Each time point represents the average of three replicate qPCR reactions from three separate experiments (n = 9).

(C and D) Comparison of the temporal profiles of 5-T, 7-T, 7-P, and 9-P between wild-type control and host males in DD (C) and between host and visitor males in DD (D). Shown are values calculated from a 3 hr moving average ± SEM (n = 27 to 45 per time point). Asterisks (red) indicate significant differences between genotypes or treatments as determined by a two-sided Student's t test (**p < 0.01; *p < 0.05). WT, wild-type; WT DD, filled blue circles; host DD, filled magenta squares; visitor DD, filled orange diamonds.

for all four compounds: $r = 0.33$ (n.s.) for 5-T, $r = 0.07$ (n.s.) for 7-T, $r = 0.22$ (n.s.) for 7-P, $r = 0.22$ (n.s.) for 7-P, and $r = -0.39$ (n.s.) for 9-P. In LD, the effects of social interactions on the level of pheromone expression in hosts persist but are subtler, predominately occurring around the time of light transitions (Figure S6). Thus, these findings demonstrate that social context has significant effects on both the overall levels of these cuticular pheromones, as well as on their temporal patterns.

Notably, the relationship between these pheromones in visitors versus homogeneous per^0 controls (Figure S7) is similar to that of the hosts and their controls (Figure 5C; Figure S6A). In particular in DD, visitor levels of 5-T and 7-T were elevated during the late subjective night and late subjective day, whereas 7-P and 9-P differed around the transitions (Figure S7A). The visitor pattern correlated inversely with per^0 controls in all three compounds: $r = -0.63$ ($p < 0.002$) for 5-T, $r = -0.44$ ($p < 0.042$) for 7-T, $r = -0.48$ ($p < 0.023$) for 7-P, and $r = -0.66$ ($p < 0.001$) for 9-P. Thus, these data show that visitors are also affected by social context.

Pheromone patterns in the wild-type hosts and per^0 visitors correlate more strongly with each other than they do with their genotypic controls in DD: $r = 0.69$ ($p < 0.001$) for 5-T, $r = 0.52$ ($p < 0.015$) for 7-T, $r = 0.46$ ($p < 0.03$) for 7-P, and $r = 0.74$ ($p < 0.0001$) for 9-P (Figures 5C and 5D; Figure S7A). One possibility is that the similarity between hosts and visitors is due to transfer by contact. However, physical contact alone cannot

explain the altered pheromone profiles shown in heterogeneous groups (Figures 5C and 5D) because both hosts and visitors display similar changes in pheromone accumulation over time; i.e., loss or gain of a compound cannot be solely explained by contact-mediated transfer if both groups are increasing or decreasing relative to controls.

Interestingly, although the levels of *desat1* transcript are reduced in hosts compared to controls, the pheromonal patterns we observed are variable: patterns of 7-P and 9-P accumulation are consistent with the reduced level of the *desat1* transcript but 5-T and 7-T are higher in hosts than controls. Despite this apparent inconsistency, the total accumulation of monounsaturated compounds is reduced in hosts [38]. Thus, the level of *desat1* expression accurately predicts the accumulation of the combined total of all monounsaturated compounds. This is consistent with the broad effect on pheromone expression observed in hypomorphic *desat1* mutant flies.

These results demonstrate that heterogeneous and homogeneous male groupings differentially affect the production of male pheromones as well as their expression on the cuticle surface, thereby illustrating the sensitivity of these traits to social context. Thus, the pheromonal blend expressed on the cuticle appears to reflect social experience.

Social Context Affects Mating Frequency

As shown above, wild-type male expression levels of specific cuticular hydrocarbons change when the flies are housed with *per*^{0} mutants, but what is the behavioral significance of these changes? We next investigated the effect of heterogeneous social context on mating behavior. We developed an assay in which six virgin males are housed with six virgin females and allowed to interact continuously over a 24 hr period (Figure 6A). We tested both homogeneous groups composed of six wild-type males with six wild-type females and mixed groups of four wild-type host and two *per*^{0} visitor males with six wild-type females. Both the temporal distribution and overall number of matings (copulations) were recorded in these different contexts.

We plotted the cumulative matings of the homogeneous and mixed groups over the 24 hr observation period (Figure 6B). Both groups mated at nearly the same rate until around CT21, when the mating frequency began to slow in the homogeneous group but not among the heterogeneous group. The two groups expressed similarly shaped temporal distributions of mating, but their levels of mating are significantly different and this difference becomes evident around dawn (CT0; Figure 6B).

After 24 hr, the total number of matings in the heterogeneous groups was 22% higher than in the homogenous groups (Figure 6C). In the heterogeneous groups, the two *per*^{0} visitor males performed a mere 5% of the matings (Figure 6C), meaning that the four wild-type host males individually performed nearly twice as many matings (1.7 times more) as their counterparts in the homogenous groups (Figure 6D). A corresponding increase in the average number of matings per female within the mixed groups was also observed (Figure 6E). Interestingly, the four wild-type males in a mixed group mated significantly more (2 more matings per male over 24 hr) than did four wild-type males in a homogeneous group of four overall (Figure 6D). Thus, the increased wild-type mating frequency is dependent on the presence of the *per*^{0} males and does not result from a difference in the number of wild-type males present in the mating chamber.

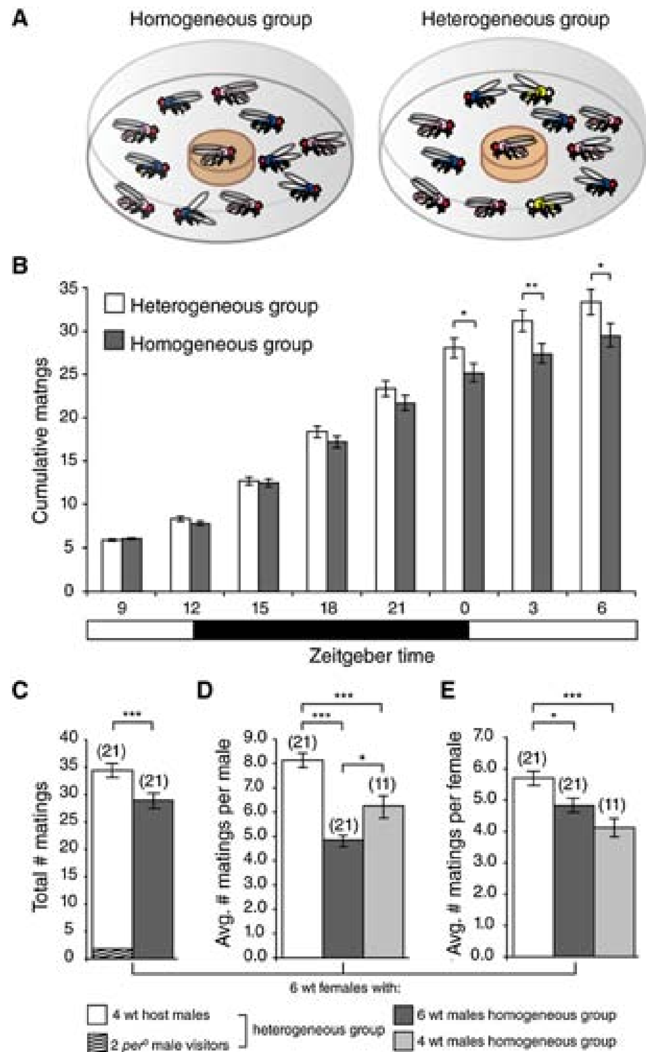

Figure 6. Social Context Changes the Amount and Temporal Distribution of Mating

(A) Schematic representation of the social-mating assay. The homogeneous group consists of six wild-type (WT) females (pink) and six WT males (blue). The heterogeneous group consists of six WT females, four WT host males, and two *per*^{0} mutant visitor males (yellow).

(B) Cumulated number of matings over a 24 hr observation period shown at 3 hr interval time points. n = 13 for both heterogeneous (white) and homogeneous (gray) groups. (ANOVA: *p < 0.05, **p < 0.01; after False Discovery Rate correction at $q = 0.1$.)

(C) Total number of matings in a heterogeneous genotype (white) or homogeneous (gray) group. ***p < 0.001, paired t test. Number of repeats (n) is between parentheses.

(D) Mean number of matings *per* wild-type male in the indicated social context. Values represent the number of wild-type male matings over 24 hr divided by the number of wild-type males present (either 4 or 6) averaged across independent experiments. ANOVA: *p < 0.05; ***p $\leq$ 0.001.

(E) Mean number of matings per wild-type female in the indicated social context. ANOVA: **p < 0.01; ***p < 0.001. Error bars indicate ±SEM in all panels.

Discussion

Social experience can influence behavior in *Drosophila*. In many cases, this influence is communicated via chemical cues, possibly in the form of pheromones [1–4, 8, 10]. Individuals sense these social cues and respond to one another as

they participate in group activities. Understanding how individuals send, receive, analyze, and respond to these signals is key to understanding how group dynamics affect behavior. By using measures of gene expression within the head and oenocytes, sex-pheromone accumulation on the outer cuticular surface, and mating behavior, we have identified aspects of a mechanism driven by social experience that influences sexual behavior in *D. melanogaster*. Our approach to studying group dynamics has reduced questions about social experience to questions about how molecular and cellular mechanisms mediate the effects of social interactions on individuals.

Regulation of Pheromone Synthesis and Display by a Circadian Mechanism

We demonstrate that *desat1* is a circadian output gene of a peripheral clock contained in the oenocytes (Figures 2 and 4). The presence of *desat1* RNA and protein in the oenocytes supports the role of these cells as the primary site for the production of sex pheromones. Circadian fluctuations in the temporal display of many cuticular hydrocarbon compounds have been previously observed [39]. Here we examine four of these compounds that act as sex pheromones. Consistent with the cyclic expression of *desat1*, the temporal profile of these pheromones are *per* dependent (Figure 3) and appear to be influenced by a peripheral clock (Figure S3). Given the unique role oenocytes play in the production of sex pheromones [6], we suggest that the temporal fluctuations in male sex-pheromone production are regulated by the oenocyte clock via the circadian regulation of *desat1*.

Environmental inputs such as humidity and temperature are known to regulate hydrocarbons in *Drosophila* [40, 41]. We show here that social interactions regulate hydrocarbon physiology. Such input from the physical and social environment may be mediated by hygro-, thermo-, and olfactory receptors in the antennae [42] or by gustatory receptors in tarsi and proboscis [43]. Whereas circadian clocks regulate olfactory [44] and possibly gustatory input, as well as oenocyte output (this study), it will be important to determine whether these clocks are required for the regulation of cuticular hydrocarbon as the fly adjusts to its environment in general.

In particular, why should sex-pheromone display be under circadian regulation? In *Drosophila melanogaster*, sex pheromones differ between the sexes and are involved in mate recognition and preference [6]. As such, these compounds represent sex-specific characters that may provide a fitness benefit when displayed at a particular concentration, in a particular blend, at specified times, and/or under certain conditions [7, 35]. All of these factors have been shown in various species to come into play when attempting to attract a mate [37, 45]. That sex-pheromone production and display are clock controlled implies that courtship and mating may have a temporal structure. Indeed, daily rhythmicity in courtship behavior was demonstrated by Hardeland some years ago [19]. More recently, others have extended these studies and have shown a temporal pattern in both courtship and mating [8, 18, 46].

Sexually dimorphic and species-specific pheromones allow for mate recognition and effective mating strategies, and so may the circadian regulation of pheromones. In this way circadian changes in pheromonal profiles may represent the means of creating a temporal niche by influencing the probability of copulation In this regard, *per* has been associated with the temporal control of when different *Drosophila* species prefer to mate, thus creating a temporal barrier in mating [18]. With this in mind, it will be important to determine whether the sex pheromones of other *Drosophila* species are also under circadian regulation, and whether the patterns differ from that of *D. melanogaster*. This might represent a mechanistic basis to the proposal of Tauber et al. [18], whereby populations exhibiting different pheromonal levels at different times might become reproductively isolated.

Social Effects on Pheromone Synthesis and Reproductive Behavior

We show that social interactions influence the circadian regulation of male sex pheromones and the mating behavior (Figures 5 and 6). The overall levels of male sex pheromones as well as their temporal patterns are affected by social context. The mixture of two genotypes (WT and per^0) within the group produced a general decrease in the total amount of monounsaturated hydrocarbon (except for 5-T and 7-T, which increase; Figure 5) and an increase in the frequency of mating. Given the prominent role male sex pheromones play in courtship behavior, it is possible that these changes in male pheromones directly relate to the change in mating behavior. 7-T is the most abundant of male sex pheromones in *D. melanogaster* and has been shown to increase female receptivity and repress male-male courtship. Males with elevated levels of 7-T show decreased latency to copulation and a higher mating success rate [47, 48]. Although an increase in 7-T may account for the increase in the frequency to copulate in our experiments, we favor an alternative explanation that a blend of hydrocarbons is associated with both the communication of social information and the influence on sexual behavior. We infer this because of the broad changes in cuticular hydrocarbons we observe between different social contexts. It remains to be determined how a change in pheromone expression in response to social interactions affects mating behavior in our social assay.

Consistent with the circadian changes in sex-pheromone expression, social interactions also affected the molecular rhythm of the oenocyte clock and the expression of *desat1*. The amplitude of *tim* and *Clk* expression decreased, whereas that of *per* increased in response to the heterogeneous social grouping; the period and the phase of the molecular rhythms remained unchanged. Although the oenocyte clock remains rhythmic, the relationship between the expression levels of these core clock genes appeared altered. Transcriptional rhythms of the clock genes contribute to clock function [49], and changes in the amplitude of transcription have been shown to affect daily locomotor activity rhythms [21]. Likewise, a change in the amplitude of clock gene expression within the oenocytes as a response to social interactions may drive the observed changes in sex-pheromone display. Correlated to the affect on *Clk* expression, the level of *desat1* RNA was also reduced, thereby providing a putative mechanism whereby social experience can affect the circadian production of sex pheromones.

The patterns of clock gene expression within tissues of the head were also affected by social interactions. The expression level of *per* and *Clk* was decreased, *tim* remained unchanged, and again, the period and phase of clock gene expression was unaffected. The head contains multiple circadian oscillators, including the central clock cells, which are required for the generation of locomotor rhythms. Although whole-head preparations prevent us from localizing this effect to only the central clock, the changes in gene expression are intriguing in the context of our previous findings, demonstrating an effect of social experience on locomotor activity rhythms [1].

Together with the effect on the oenocytes, it would appear that social interactions affect multiple circadian systems, including both the central and peripheral clocks. Notably, these observations indicate that the amplitude of clock gene expression plays an important role in modulating both physiological (e.g., pheromone production) and behavioral (e.g., locomotor activity) rhythms.

A direct demonstration of the mechanistic links between the individual observations presented in this study (i.e., oenocyte clock > *desat1* rhythm > pheromone rhythm > influences mating behavior) requires a means to manipulate the oenocyte clock specifically and the rhythmic expression *desat1*. This could be achieved through the use of the Gal4/UAS system targeting the oenocytes. However, it must be noted that the expression patterns of Gal4 driver lines used previously to examine the function of oenocytes are not restricted to only the oenocytes [26, 28, 29]. In some cases, expression was observed in the brain and/or fat body, two tissues known to affect courtship and circadian behavior [50]. Although there is no doubt about the use of these reagents to manipulate pheromones, we conclude that the available oenocyte drivers are not adequate to discriminate oenocyte-specific effects on behavior or the temporal pattern of pheromone accumulation (Figure S1).

Indirect Genetic Effects

It is unlikely that social communication in *Drosophila melanogaster* is limited to chemical signaling; indeed, tactile and auditory displays are important features of reproductive behavior [51], and it seems likely that a variety of sensory modalities are linked to pathways that mediate social responses [52]. However, our data suggest that pheromonal responses are extremely sensitive to the social environment.

The possibility that behavioral feedback regulates molecular physiology in the fruit fly was proposed nearly two decades ago in the context of circadian rhythms [53]. Interestingly, the same idea has been proposed in a quantitative theory that views the social environment as a selective pressure. This theory of indirect genetic effects relies on the idea that social interactions, occurring over generations, may direct the distribution of alleles within a population [54]. According to this view, the relationship between an individual's phenotype and genotype is shaped in part by social context. We have observed this here. Given the increase in mating associated with a mixed social grouping (Figure 6), the influence of social interactions on clock gene and *desat1* expression may represent a detailed example in which an indirect genetic effect represents a modification in gene expression. Our data may offer a glimpse into the effects of social interactions on the mechanisms of inheritance. The ability to quantify the effects of social and physical environmental influences on behavior, together with the powerful tools available for studying inherited mechanisms of behavior, suggests that *Drosophila melanogaster* will be an important model organism for understanding the evolution of sociality.

Experimental Procedures

Fly Strains

Flies were reared on medium containing agar, glucose, sucrose, yeast, cornmeal, wheat germ, soya flour, molasses, propionic acid, and Tegosept in a 12 hr light/dark cycle (LD 12:12) at 23°C and 70% humidity. Male and female flies were anesthetized with CO_2 and separated within 8 hr of eclosion. For all quantitative PCR analyses, with the exception of those performed in the context of the social assay, male pairs (setup in multiple) were raised in individual glass vials (10 mm diameter × 75 mm height) containing 1 ml medium, entrained for 4 days in LD 12:12 conditions, and sacrificed the fourth day after eclosion. To examine free-running oscillations in gene expression, flies were placed in constant dark (DD) conditions at the end of the third day of entrainment, and sacrificed on the first full day of DD conditions.

Experiments examining the effects of social interactions utilized a modified version of the assay described by Levine et al. [1]. Social treatments consisted of 32 Canton-S (host) males housed with 8 *y,per^{01},w* (visitor) males. Control groups consisted of either 40 Canton-S or 40 *y,per^{01},w* males. Flies were housed in plastic vials (25 mm diameter × 92 mm height) containing 10 ml medium and were entrained for a total of 6 days under LD 12:12 conditions; on the fourth day, 32 Canton-S flies were mixed with 8 *y,per^{01},w* flies, maintained in LD 12:12 for 2 additional days, and placed into DD on the sixth day. For quantitative PCR experiments, flies were sacrificed on the second full day of DD. For analysis of pheromone levels, flies were sacrificed on the first full day of DD conditions.

Canton-S strain was used as the wild-type strain. The *y, per^{01},w,* and *per7.2:2* are described in Levine et al. [1]. The 1407-Gal4 line is described in Ferveur et al. [26]. The *desat1$^{1573\text{-}1}$* and *desat1$^{1573\text{-}N2}$* lines are described in Marcillac et al. [36]. UAS-mCD8::GFP and UAS-NZ.lacZ were obtained from the Bloomington Stock Center.

Quantitative RT-PCR

Oenocytes were collected from five male flies over a period of ~2 hr, at eight scheduled time points spanning a 24 hr period. RNA was isolated from dissected oenocyte preparations (see Supplemental Experimental Procedures) with the RNeasy Micro kit (QIAGEN), and total RNA was reverse transcribed with the StrataScript QPCR cDNA Synthesis kit (Stratagene). Quantitative PCR reactions were performed with the qPCR MasterMix Plus for SYBR Green I kit (Eurogentec), on an ABI PRISM 7700 Sequence Detection System. The relative level of gene transcript expression was determined separately for *timeless*, *period*, *Clock, desat1,* and *Rp49* from cDNA prepared from a common pool of dissected oenocytes. *Rp49* served to normalize gene expression. qPCR reactions were performed in triplicate, and the specificity of each reaction was evaluated by dissociation curve analysis. Each experiment was replicated three times.

Pheromone Extraction

Pheromone extracts were obtained from Canton-S and *y,per0,w* flies every hour over a 24 hr period. Extracts were obtained from three individuals per time point, selected from a vial containing 40 flies. Flies were removed under either light or red-light conditions depending on light schedule, and anesthetized with ether. Each fly was placed into an individual glass microvial containing 50 μl of hexane containing 10 ng/μl of octadecane (C18) and 10 ng/μl of hexacosane (nC26) as injection standards. To achieve efficient extraction, the microvials were gently agitated for 5 min. Flies were removed with a thin wire probe, and the extracts were stored at −20°C prior to analysis. Experiments were repeated from 3 to 5 times per treatment. Identical methods were used to obtain pheromone extracts from *per7.2:2* and the *desat1^{1573}* lines. Extracts were examined by gas chromatography (see Supplemental Experimental Procedures).

Mating Assay

Testing arenas consisted of 60 × 15 mm plastic Petri dishes. A cylinder of fly flood (22 × 5 mm diameter) was placed in the center of the testing arena. Six Canton-S virgin females were aspirated into the testing arena followed a few minutes later by six males (six *Canton-S* males or four *Canton-S* plus two *y,per^{01},w* males). Flies were from 5- to 6-day-old. Group interactions were recorded with Northern Eclipse software (Version 7.0 from Empix Imaging Inc.) set to take time-lapse images at 2 min intervals for 24 hr. The collected images were scored for the time at which copulation events occurred and strain of the male involved. Experiments were started at CT8 and groups were tested in LD 12:12 with constant red light (680 nm) illumination, allowing for copulation events to be monitored in darkness.

Statistical Analyses

To determine whether RNA expression patterns are cyclic, the *R* statistical language was used to perform nonlinear best cosine curve fitting on combined RNA relative expression data (see Supplemental Experimental Procedures). Relative expression amounts were calculated with the REST relative expression method [55] with *Rp49* as an internal reference gene. Pheromone expression levels were determined as previously described [39]. To determine whether pheromone levels differed at a given time interval, we

performed two-tailed Student's t tests. ANOVA followed by the post-hoc Tukey-Kramer test was used to assess significance in the mating behavior.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/18/1373/DC1/.

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