Flexible origin of hydrocarbon/pheromone precursors in Drosophila melanogaster

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Abstract
In terrestrial insects, cuticular hydrocarbons (CHCs) provide protection from desiccation. Specific CHCs can also act as pheromones, which are important for successful mating. Oenocytes are abdominal cells thought to act as specialized units for CHC biogenesis that consists of long-chain fatty acid (LCFA) synthesis, optional desaturation(s), elongation to very long-chain fatty acids (VLCFAs), and removal of the carboxyl group. By investigating CHC biogenesis in Drosophila melanogaster, we showed that VLCFA synthesis takes place only within the oenocytes. Conversely, several pathways, which may compensate for one another, can feed the oenocyte pool of LCFAs, suggesting that this step is a critical node for regulating CHC synthesis. Importantly, flies deficient in LCFA synthesis sacrificed their triacylglycerol stores while maintaining some CHC production. Moreover, pheromone production was lower in adult flies that emerged from larvae that were fed excess dietary lipids, and their mating success was lower. Further, we showed that pheromone production in the oenocytes depends on lipid metabolism in the fat tissue and that fatty acid transport protein, a bipartite acyl-CoA synthase (ACS)/FA transporter, likely acts through its ACS domain in the oenocyte pathway of CHC biogenesis. Our study highlights the importance of environmental and physiological inputs in regulating LCFA synthesis to eventually control sexual communication in a polyphagous animal. —Wicker-Thomas, C., D. Garrido, G. Bontonou, L. Napal, N. Mazuras, B. Denis, T. Rubin, J-P. Parvy, and J. Montagne. Flexible origin of hydrocarbon/pheromone precursors in Drosophila melanogaster. J. Lipid Res. 2015. 56: 2094–2101.

Supplementary key words fatty acid metabolism • genetics • nutrition • gene expression • lipid droplets • lipoprotein receptors • triglycerides • homeostasis

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Organis of multicellular organisms complete specific metabolic functions that integrate at the physiological level to maintain body homeostasis. The integument is one such organ (1). It creates an external barrier to protect against environmental injuries. In mammals, skin can be permeable if there are defects in lipid synthesis and/or transport (2). The integument in small animals requires even greater resistance to desiccation because of their greater surface/volume ratio (3).

In terrestrial insect species, the integument is covered by molecules derived from lipids, including triacylglycerols (TAGs), waxes, and cuticular hydrocarbons (CHCs) (4, 5). CHCs provide protection from desiccation. They may also act as pheromones, where the chain length, the structure, and the position of double bonds in specific CHCs play a key role in sexual communication (6). In Drosophila melanogaster, 7-tricosene (7-C23:1) is the main male pheromone, and 7,11-dienes are the female pheromones, the main being 7,11-heptacosadiene (7,11-C27:2) (7).

CHCs are formed by FA reduction to aldehyde, followed by oxidative decarbonylation (8) (Fig. 1A). In eukaryotic cells, FA synthesis first requires acetyl-CoA carboxylase (ACC) to catalyze the synthesis of malonyl-CoA, the rate-limiting metabolite (9). Next, FASN successively incorporates a number of malonyl-CoA units on an acetyl-CoA primer to form long-chain FAs (LCFAs) (10). Very long-chain FA

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Abbreviations: ACC, acetyl-CoA carboxylase; ACS, acyl-CoA synthase; CHC, cuticular hydrocarbon; FATP, fatty acid transport protein; FB, fat body; GFP, green fluorescent protein; HADC, 3-hydroxyacyl-CoA-dehydratase; KAR, 3-ketoacyl-CoA-reductase; LCFA, long-chain fatty acid; LD, lipid droplets; LpR, lipophorin receptor; mb, methylated/branched; RNAi, interfering RNA; TAG, triacylglycerol; TER, transenoyl-CoA-reductase; VLCFA, very long-chain fatty acid.

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(VLCPA) synthesis requires four distinct enzymes to further elongate a fatty acyl-CoA primer (11). A member of the elongase family, whose various gene products differ from one another in their tissue-specific expression and substrate specificity, catalyzes the process of incorporating malonyl-CoA (12). The subsequent steps are successively catalyzed by a 3-keto-acyl-CoA reductase (KAR), a 3-hydroxy-acyl-CoA dehydratase (HADC), and a trans-enoxy-CoA reductase (TER). Desaturating enzymes can operate between elongation steps, leading to unsaturated FAs (11).

Genetic studies using the D. melanogaster model have identified several enzymes that are required for CHC biogenesis in specific abdominal cells called oenocytes (8, 13–18). More recently, it has been shown that FASN<sup>CG13774</sup>, one of the three Drosophila FASN orthologs, is specifically expressed in the oenocytes for the synthesis of methylated/branched (mb) FAs, which are precursors of 2-methylalkanes (mbCHCs) (19). This study also revealed that FASN<sup>CG17374</sup> is expressed in the oenocytes, whereas FASN<sup>CG3523</sup>, which was expected to be ubiquitously expressed (20), is excluded from the oenocytes but is present in the fat body (FB; the organ in charge of hepatic and adipose functions) (19). Together, these studies support the notion that the entire metabolic pathway sustaining CHC production takes place within the oenocytes.

To comprehensively address this issue, we used D. melanogaster genetics and performed systematic knockdown in the oenocytes of several enzymes that cover the entire process of FA synthesis. We showed that LCFA elongation to VLCPA takes place within the oenocytes. In contrast, LCFA synthesis-deficient flies still produce CHCs, though in reduced amounts, indicating that dietary lipids may partly compensate for LCFA deficiency. Further, we observed that providing fat-enriched food during larval development impedes pheromone biosynthesis in adult flies and decreases their mating success. Finally, we showed that FA homeostasis may influence CHC biogenesis and identified a putative acyl-CoA synthase required for CHC synthesis within the oenocytes.

Fig. 1. Oenocyte-targeted knockdown of enzymes required for VLCPA synthesis. (A) Scheme of the CHC biosynthetic pathway. Metabolites are indicated in black, and enzymes in blue. ELOVL, elongase; CPR, NADPH-cytochrome P450 reductase; CYP4G1, aldehyde oxidative decarbonylase P450. (B, B’) Green fluorescent protein (GFP) gase; CPR, NADPH-cytochrome P450 reductase; CYP4G1, aldehyde metabolites are indicated in black, and enzymes in blue. ELOVL, elongase family, whose various gene products differ from one another in their tissue-specific expression and substrate specificity, catalyzes the process of incorporating malonyl-CoA (12). The subsequent steps are successively catalyzed by a 3-keto-acyl-CoA reductase (KAR), a 3-hydroxy-acyl-CoA dehydratase (HADC), and a trans-enoxy-CoA reductase (TER). Desaturating enzymes can operate between elongation steps, leading to unsaturated FAs (11).

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**MATERIALS AND METHODS**

Fly maintenance and genetics

Flies were maintained on standard media, except for the analysis of the FASN mutant raised on a low carbohydrate media supplemented with dietary lipids as previously described (21). The interfering-RNA (RNAi) fly lines provided from Fly Stocks of the National Institute of Genetics (NIG-FLY) or the Vienna Drosophila Resource Center (VDRC) (22) are listed (supplementary Table 1), except FASN<sup>CG17374</sup>-RNAi (23). Lipophorin receptor (LpR)/1/2 deficiency was generated as previously described (23) using the P Bac[PB1c02106 and P[XP]d10508 elements (24). The da-gal4 (Bloomington stock center), BG-gal4 (25), 1407-gal4 (13), Mex-gal4 (26), and C564-gal4 (27) drivers express the Gal4 transcription factor ubiquitously, in larval oenocytes, in pupal and adult oenocytes, in the gut, and in the FB, respectively. Drivers were maintained as heterozygotes over a Cyo or a TM3 balancer or the cosegregating SM5/TM6 balancers. In all RNAi knockdown experiments, balanced gal4-driver flies were crossed to RNAi flies.

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In their progeny, RNAi-expressing flies contain the gal4 driver, whereas control flies contain the balancer chromosome.

Biochemical analysis

The C23-C29 CHCs, which are synthesized after adult eclosion (7), were extracted from 4- to 5-day-old flies and analyzed by gas chromatography as previously described (16). Control and test flies were issued either from the sibling progeny of the same crosses (RNAi lines and LpR mutants) or from flies reared at the same time in the same conditions (nutrition test on control and FASN mutants). At least 10 flies were analyzed for each genotype. TAG measurements and quantitative RT-PCR were performed from four replicates of three adult males, as previously described (21).

Histochemistry

The dorsal parts of abdominal cuticles were dissected from 4- to 5-day-old flies in PBS, fixed for 20 min at room temperature in 4% paraformaldehyde, and then washed three times in PBS. Lipid (Nile Red) and nuclear (TO-PRO-3-iodide) staining was performed as previously described (23). Cuticles were mounted in DABCO and examined using a Nikon (TE-2000-U) confocal microscope.

Fly behavior

Desiccation tests and mate choice tests were performed as previously described (8, 28).

RESULTS

Elongation of LCFA to VLCFA takes place within the oenocytes

To investigate the CHC biosynthetic pathway in *D. melanogaster*, we first evaluated the ACC requirement. The 1407-gal4 driver that expresses Gal4 in the oenocytes from the late third larval stage (Fig. 1B) was used to direct an RNAi to the unique ACC *Drosophila* gene (hereafter called *Oe-ACC-RNAi*). In these flies, CHCs were almost fully depleted (Fig. 1C; supplementary Table 2A, B). This defect was a direct consequence of ACC knockdown in the oenocytes: expression of *svp-gal80* (25), which specifically blocks Gal4 activity in the oenocytes (Fig. 1B, B’), completely rescued CHC production (Fig. 1D; supplementary Table 2A, B).

Next, we evaluated the requirement for enzymes that catalyze VLCFA synthesis. The *D. melanogaster* genome encodes 20 elongase members (29). Stringent in silico analyses indicate that KAR is encoded by seven putative genes, whereas control flies contain the balancer chromosome.

**Functional roles of the oenocyte-specific FASN genes**

To evaluate the role of LCFA synthesis in the oenocytes, we induced RNAi to FASN*CG17374* and FASN*EG17374*, the two FASN genes reported to be expressed in the oenocytes (19). Consistent with the study of Chung and colleagues (19), we observed a dramatic reduction in mbCHCs in *Oe>FASN*CG17374* RNAi flies, although the total amount of CHCs did not change (Fig. 2A–C; supplementary Table 3A, B). In contrast, RNAi to FASN*EG17374* did not affect CHC levels (Fig. 2D–F; supplementary Table 3A, B).

FASN*CG17374* has been proposed to act in maintaining ecological isolation between two *Drosophila* species through desiccation resistance and effects on mating choice (19). Therefore, we investigated these functions in *D. melanogaster*. *Oe>ACC-RNAi* flies were extremely sensitive to desiccation (Fig. 2G), a phenotype suppressed when coexpressing the *svp-gal80* transgene (supplementary Fig. 1A). Further, *Oe>FASN*CG17374* RNAi flies were not sensitive to desiccation, whereas *Oe>FASN*EG17374* -RNAi flies were moderately sensitive (Fig. 2G; supplementary Table 4). The desiccation sensitivity of *Oe>FASN*CG17374* -RNAi flies suggests that FASN*CG17374* catalyzes the synthesis of precursors for other FA derivatives, potentially waxes or TAGs (5), that are required to secure cuticular watertightness. The desiccation sensitivity of *Oe>ACC-RNAi* flies may result from a default in CHCs and/or in these non-CHC lipid derivatives. Nonetheless, oenocyte expression of an RNAi to *Cyp4g1*, which specifically catalyzes decarbonylation to CHC (8), resulted in desiccation sensitivity similar to that of *Oe>ACC-RNAi* flies (supplementary Fig. 1B, B’). These findings indicate that, although non-CHC lipid derivatives appear to be required to produce an efficient cuticle, CHC depletion is sufficient to fully impair cuticular watertightness.

Next, we investigated whether FASN*CG17374* or FASN*EG17374* knockdown affects mating choice in *D. melanogaster*. Single wild-type (Canton-S) females did not exhibit any preference when given a choice between Canton-S males and one of either genotype *Oe>FASN*CG17374* -RNAi or *Oe>FASN*EG17374* -RNAi (Fig. 2H). Reciprocally, single Canton-S males did not exhibit any preference when given a choice between a Canton-S female and a female of either genotype *Oe>FASN*CG17374* -RNAi or *Oe>FASN*EG17374* -RNAi (Fig. 2H). Although we cannot anticipate the consequence of total mbCHC depletion, our findings indicate that a 50% reduction in mbCHCs does not affect desiccation resistance or sexual recognition in *D. melanogaster*.
To get further insights into the requirement of FA synthesis in CHC biogenesis, we took advantage of a mutant \( \text{FASN}^{G^{24-23}} \) that removes both \( \text{FASN}^{CG3524} \) and \( \text{FASN}^{CG3523} \) genes. This mutant is lethal at the L1 stage but can be rescued by an appropriate lipid-supplemented diet (21). We also induced \( \text{FASN}^{G^{17374}} \)-RNAi with the 1407-gal4 driver in \( \text{FASN}^{G^{24-23}} \) mutants (\( \text{FASN}^{G^{24-23};CG17374i} \)). We focused on mutant males because they survived better than females after eclosion. Importantly, both control and mutant animals were raised on the lipid-supplemented media during larval development. However, newly emerged flies were transferred to standard food for 4 days because adult flies tend to stick to the lipid-supplemented media, leading to a high rate of lethality. Surprisingly, control males emerging from larvae fed a lipid-supplemented diet contained roughly half the amount of all CHCs, including mbCHCs, compared with control males raised on standard media (Fig. 3A, A; supplementary Table 5A). Nonetheless, when emerged from larvae fed a lipid-supplemented diet, \( \text{FASN}^{G^{24-23}} \) mutant males contained a similar amount of CHCs compared with control males raised in the same conditions (Fig. 3A, A; supplementary Table 5B). Further, CHCs were strongly reduced, but not completely eliminated, in \( \text{FASN}^{G^{24-23};CG17374} \) males (Fig. 3A, A; supplementary Table 5B), indicating that \( \text{FASN}^{CG17374} \) can also contribute to the pool of LCFAs used for CHC biogenesis.

To evaluate how these \( \text{FASN} \) mutants consume lipid stores, we analyzed TAG levels either in 0- to 1-day-old or in 4- to 5-day-old adult flies. In contrast to CHCs (Fig. 3A, A'), TAG levels in 0- to 1-day-old flies were slightly higher in control animals fed a lipid-supplemented diet (Fig. 3B). Consistent with the higher fasting resistance of newly emerged versus 3-day-old adult flies (30), TAG stores were at very high levels at the day of eclosion and then decreased in 4- to 5-day-old males, irrespective of larval lipid supplementation (Fig. 3B). Conversely, in \( \text{FASN}^{G^{24-23}} \) and \( \text{FASN}^{G^{24-23};CG17374} \) mutants, TAG stores were dramatically lower at eclosion and almost eliminated 4 days later (Fig. 3B).

Finally, we analyzed \( \text{FASN} \) expression in flies that emerged from larvae fed a lipid-supplemented diet. Quantitative RT-PCR analysis indicated that feeding control larvae with the lipid-supplemented media led to a significant decrease in expression of the three \( \text{FASN} \) genes in 1-day-old adult males (Fig. 3C–E). Moreover, we observed that, in the presence of single Canton-S females, males that emerged from larvae fed a lipid-supplemented media had lower mating success than those raised on standard media (Fig. 3F). Furthermore, females that emerged from larvae fed a lipid-supplemented media were less attractive than those raised...
on standard media (Fig. 3F). Consistently, several female-specific pheromones were reduced in the females fed on lipid-supplemented media, while total CHCs were not significantly affected (supplementary Table 5C; Fig. 3G). Considering that CHC biogenesis occurs after eclosion (7), these findings indicate that an excess of dietary lipids during the juvenile period restrains pheromone biogenesis in adults and reduces their mating success.

**FA metabolism in the FB affects CHC biogenesis**

The observations described previously suggest that the LCFA s used for CHC synthesis may originate somewhere outside the oenocytes and, thus, must be taken up into the oenocytes before CHC biogenesis. The lipoprotein receptors LpR1 and LpR2 are expressed in the oenocytes (25). We previously showed that LpR2 was required for lipid uptake into ACC-deficient oenocytes in larvae (23). Therefore, here we monitored CHC amounts in mutants in which LpR1, LpR2, or both genes together had been deleted (Fig. 4A). CHC amounts were not affected in mutants with either LpR1 or LpR2 deletions (Fig. 4B, C; supplementary Table 6A, B) but were severely decreased in the mutants with a double LpR1/LpR2 deletion (Fig. 4D; supplementary Table 6A, B). Consistent with this, flies expressing the ubiquitous da-gal4 driver to direct LpR1-RNAi and LpR2-RNAi together had far fewer CHCs (Fig. 4E; supplementary Table 6C, E). However, CHCs remained unaffected when both RNAi were directed by either an oenocyte- or a gut-specific driver (Fig. 4F, G; supplementary Table 6C–F). Conversely, we observed a significant reduction in CHCs when directing LpR1-RNAi and LpR2-RNAi together with C564-gal4, an FB-specific driver (Fig. 4H; supplementary Table 6D, F), indicating that alteration of lipid metabolism in the FB affects oenocyte activity. Therefore, to determine whether FA synthesis in the FB plays a role in CHC biogenesis, we used the C564-gal4 driver to knockdown FASNCG3523*, the only FASN gene expressed in the FB (19). CHCs were slightly but significantly reduced in FB>FASNCG3523RNAi flies (Fig. 4I; supplementary Table 6G, H), suggesting that FA synthesis within the FB may participate in feeding the pool of LCFA s used for CHC biogenesis in the oenocytes.

**A bipartite FA transporter/acyl-CoA synthase is required in the oenocytes for CHC biogenesis**

Based on our previous study, which showed that a default in VLCFA synthesis in larval oenocytes results in tracheal defects (23), we screened RNAi to 140 genes encoding FA metabolic effectors (supplementary Table 7), using the BO-gal4 driver that is active in embryonic and larval oenocytes (25). In this way, we found that fatty acid transport protein (FATP) was essential in larval oenocytes (supplementary Table 7). Further, CHCs were almost fully depleted in Oe>fatp-RNAi flies (Fig. 5A; supplementary Table 8A, B) indicating that FATP is also required in adult oenocytes.

In addition to its FA-transporter domain, FATP also contains an acyl-CoA synthase (ACS) domain. However, FATP is unlikely to work through its FA-transporter domain, at least for the production of mbCHCs that are depleted in Oe>fatp-RNAi (supplementary Table 8A, B); some of their precursors (mbFAs) are synthesized within the oenocytes and therefore do not require transporter-mediated uptake. Finally, we investigated potential cytological defects in the oenocytes of CHC-depleted flies. In the abdomen of adult flies, oenocytes and the FB are organized as tightly associated rows of cells that can be easily distinguished (Fig. 5B–D). Analysis of lipid content revealed that the FB has a large capacity to store lipid droplets (LDs) (Fig. 5B), whereas no LDs could be detected in the oenocytes (Fig. 5C, D). Conversely, oenocytes of Oe>fatp-RNAi flies contained more LDs than did control oenocytes.
The origin of pheromone precursors in Drosophila is an important aspect of understanding sexual communication in flies. Previous studies have suggested that the entire CHC/pheromone biogenesis pathway takes place within the oenocytes, because targeted knockdown of enzymes acting either in the early or final steps of this metabolic pathway led to depleted CHCs (8, 13, 19). Here we provide evidence that VLCFA synthesis in D. melanogaster happens exclusively within the oenocytes, while there is flexibility in where the LCFAs used to feed this metabolic pathway originate (Fig. 6).

Lipid homeostasis appears to influence CHC production because FB disruption of genes encoding lipid metabolic effectors (FASN, LpR1, and LpR2) decreases the amount of CHCs. It has been previously reported that providing lipid-enriched food to the larvae of the cactophilic Drosophila mojavensis decreases CHC production in adults (31). Here, we show that this is also the case in the polyphagous D. melanogaster and that the decrease in CHCs impacts sexual communication in adults. Because CHCs are synthesized after adult eclosion (7), it is tempting to speculate that a larval nutritional signal modulates the competence for CHC biogenesis in adult oenocytes. Alternatively, reduced CHCs induced when dietary lipids are provided during the larval stages may directly depend on repression from circulating lipids that could remain higher in the resulting adult flies. This repression operates at least in part on the FASN gene transcription, including FASN CG3523 that is not active in the oenocytes. However, further experiments will be required to determine whether this repression results from a developmental event that is induced early or from a direct effect due to higher lipid content.

In an attempt to identify novel genes critical for oenocyte function, we have identified fatp as an essential gene in larval oenocytes and further showed that fatp is required for CHC synthesis in adult oenocytes. Previous Drosophila studies on fatp mutants reported metabolic and eye-specific phenotypes (32, 33) but did not discriminate between FA transporter or ACS activities. Importantly, our observations favor the notion that in the process of CHC synthesis, FATP acts instead through its ACS domain, because fatp knockdown impedes mbCHC synthesis in the oenocytes, and mbCHC precursors are mainly synthesized within the oenocytes. Further, we observed a dramatic accumulation of LDs in ACC-deficient oenocytes (Fig. 5F) and to a lesser extent in KAR CG3523-deficient oenocytes (Fig. 5G). Importantly, the dramatic accumulation of LDs observed in oenocytes of Oe>ACC-RNAi flies was still visible when coexpressing fatp-RNAi (Fig. 5H). Accumulation of LDs may be due to either an increase in lipid uptake or a decrease in CHC synthesis and a subsequent accumulation of precursors. Nonetheless, in ACC-deficient oenocytes, there is no malonyl-CoA and therefore no FA synthesis. Thus, the accumulation of LDs must be due to an increase in lipid uptake that does not depend on FATP.

**DISCUSSION**

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Further, in the mouse, skin-targeted knockout of FATP4 provokes watertightness defects that can be rescued by transgenic overexpression of wild-type FATP4, but not of a variant containing two point mutations in its ACS domain (35). Therefore, because any FA modification first requires CoA esterification (8), the phenotype induced by fatp knockdown suggests that FATP is required within the oenocytes through its ACS domain, rather than its FA-transporter domain (Fig. 6). In summary, our findings support the notion that lipid uptake into the oenocytes does not involve LpR1, LpR2, or FATP. Therefore, identification of other candidates must be undertaken to determine whether oenocyte lipid uptake proceeds through lipoprotein particles or free FAs.

Our findings highlight the existence of compensatory processes that regulate the production of CHCs. In Dro sophila serrata, the synthesis of mbLCFAs used as mbCHC precursors takes place solely within the oenocytes (19). Here, we show that in D. melanogaster, oenocyte knockdown of FASN CG3524 also results in a strong reduction of mbCHCs. However, FASN/H900424-23 mutants that do not have the FASN CG3524 gene still produce mbCHCs, demonstrating that the mbLCFAs used for mbCHC synthesis can be either synthesized within the oenocytes or provided by dietary lipids. Compensatory processes are also highlighted by oenocyte disruption of the FASN CG17374 gene, which does not affect CHC amounts unless it is produced in an FASN/H900424-23 mutant. This suggests either that oenocyte lipid uptake proceeds through lipoprotein particles or free FAs.

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