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.**

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Organs 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

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-hydroxy-acyl-CoA-dehydratase; KAR, 3-keto-acyl-CoA-reductase; LCFA, long-chain fatty acid; LD, lipid droplets; LpR, lipophorin receptor; mb, methylated/branched; RNAi, interfering RNA; TAG, triacylglycerol; TER, *trans*-enoyl-CoA-reductase; VLCFA, very long-chain fatty acid.

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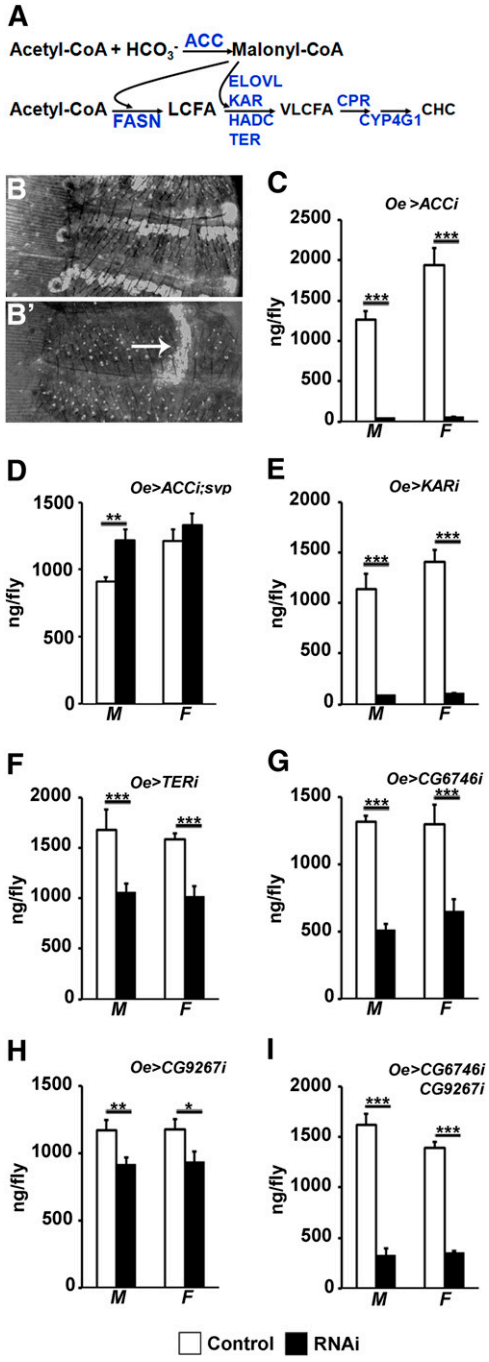

Fig. 1. Oenocyte-targeted knockdown of enzymes required for VLCFA synthesis. (A) Scheme of the CHC biosynthetic pathway. Metabolites are indicated in black, and enzymes in blue. ELOVL, elongase; CPR, NADPH-cytochrome P450 reductase; CYP4G1, aldehyde oxidative decarbonylase P450. (B, B') Green fluorescent protein (GFP) fluorescence in the abdomen (side view; anterior top, ventral left) of *Oe>UAS-GFP* (B) and *svp-gal80;Oe>UAS-GFP* (B') pharates. At the end of metamorphosis, the oenocytes (B) appear as a dorsal row of cells below the tegument of each abdominal segment. Note that in *svp-gal80;Oe>UAS-GFP* pharates, the green fluorescence in the oenocytes is lost, while the gut (arrow) still expresses the GFP (B'). (C–I) Total CHCs in *Oe>ACC-RNAi* (C), *Oe>ACC-RNAi;svp-Gal80* (D), *Oe>KAR^{CG1444}-RNAi* (E), *Oe>TER^{CG10849}-RNAi* (F), *Oe>HADC^{CG6746}-RNAi* (G), *Oe>HADC^{CG9267}-RNAi* (H), or *Oe>HADC^{CG6746}-RNAi;Oe>HADC^{CG9267}-RNAi* (I) flies. In each panel, analysis was performed on males (left) and females (right); controls (empty bars) are the silent-RNAi siblings of the *Oe>RNAi* flies (black bars). Each bar represents mean ± SEM (n = 10). Significance for statistical tests: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(VLCFA) 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*-enoyl-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^{CG3524}*, 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^{CG17374}* is expressed in the oenocytes, whereas *FASN^{CG3523}*, 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 VLCFA 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.

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^{CG17374}-RNAi* (23). *Lipophorin receptor (LpR)1/2* deficiency was generated as previously described (23) using the PBac[PB]c02106 and P[XP]d10508 elements (24). The *da-gal4* (Bloomington stock center), *BO-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;TM6B* balancers. In all RNAi knockdown experiments, balanced *gal4*-driver flies were crossed to RNAi flies.

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, TER by a single gene ($TER^{CG10849}$), and HADC by two putative genes ($HADC^{CG6746}$ and $HADC^{CG9267}$) (supplementary Table 1). We focused on the strongest *KAR* homolog (KAR^{CG1444}) because we previously reported that it was required early in development for an essential function of larval oenocytes (23). In *Oe>*KAR^{CG1444}*-RNAi* animals, CHCs were almost completely eliminated (Fig. 1E; supplementary Table 2C, D), indicating that knockdown of only the KAR^{CG1444} ortholog is sufficient to largely eliminate CHC synthesis in *D. melanogaster* oenocytes. RNAi to $TER^{CG10849}$ led to a partial decrease in CHC levels (Fig. 1F; supplementary Table 2C, D), possibly because of variable efficacy in RNAi knockdown. RNAi to $HADC^{CG6746}$ led to a strong decrease in CHC levels (Fig. 1G; supplementary Table 2E, F), whereas RNAi to $HADC^{CG9267}$ led to a moderate decrease in CHC levels (Fig. 1H; supplementary Table 2E, F). However, coexpression of both RNAis decreased CHC levels even more (Fig. 1I; supplementary Table 2E, F), suggesting that both HADCs contribute to this enzymatic activity. Together, these findings indicate that VLCFA synthesis of CHC precursors takes place exclusively within the oenocytes.

Functional roles of the oenocyte-specific *FASN* genes

To evaluate the role of LCFA synthesis in the oenocytes, we induced RNAi to $FASN^{CG3524}$ and $FASN^{CG17374}$, 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>*$FASN^{CG3524}$*-RNAi* flies, although the total amount of CHCs did not change (**Fig. 2A–C**; supplementary Table 3A, B). In contrast, RNAi to $FASN^{CG17374}$ did not affect CHC levels (Fig. 2D–F; supplementary Table 3A, B).

$FASN^{CG3524}$ 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>*$FASN^{CG3524}$*-RNAi* flies were not sensitive to desiccation, whereas *Oe>*$FASN^{CG17374}$*-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 $FASN^{CG3524}$ or $FASN^{CG17374}$ 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>*$FASN^{CG3524}$*-RNAi* or *Oe>*$FASN^{CG17374}$*-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>*$FASN^{CG3524}$*-RNAi* or *Oe>*$FASN^{CG17374}$*-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*.

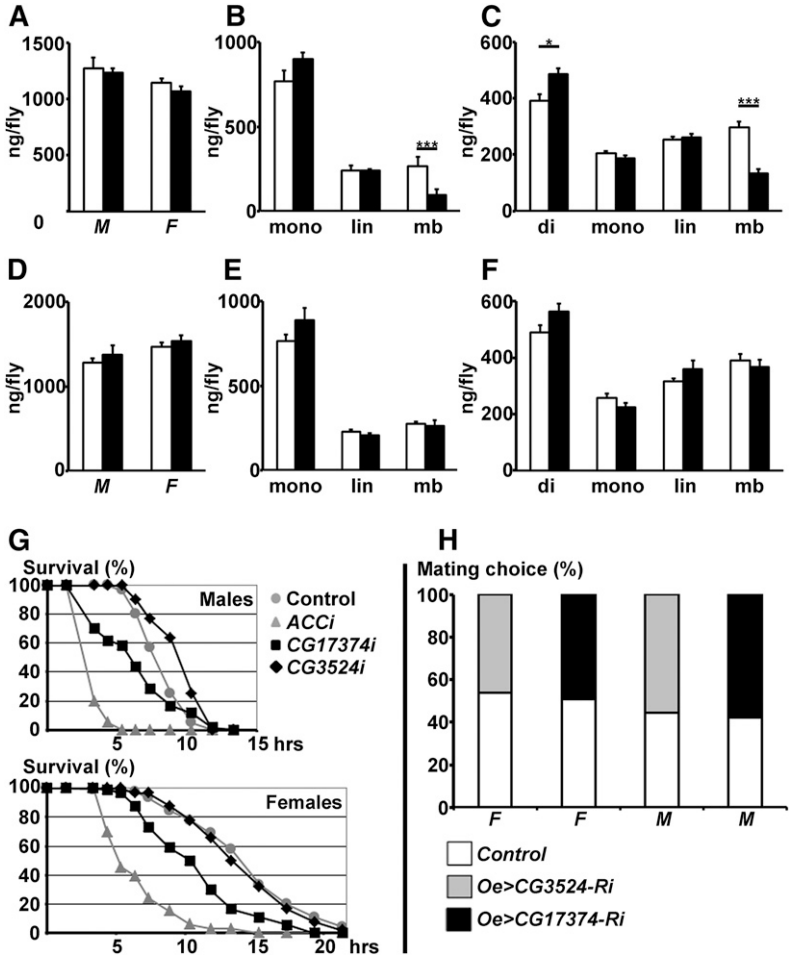

Fig. 2. Oenocyte-targeted knockdown of $FASN^{CG3524}$ and $FASN^{CG17374}$. (A–F) CHCs in *Oe>*$FASN^{CG3524}$*-RNAi* (A–C) and *Oe>*$FASN^{CG17374}$*-RNAi* (D–F) flies. (A, D) Total CHCs in males (left) and females (right). (B, C, E, F) Amounts in diene (di), monoene (mono), saturated linear (lin), and mb CHC classes in males (B, E) and females (C, F). Control and RNAi-expressing flies and statistical analyses are as in Fig. 1C–I. (G) Hours of survival to desiccation stress of male and female control flies (*gal4* driver alone) and of *Oe>ACC-RNAi*, *Oe>*$FASN^{CG3524}$*-RNAi*, or *Oe>*$FASN^{CG17374}$*-RNAi*. n = 90. Statistical analysis (supplementary Table 4) indicates that the survival rates of *ACC-RNAi* and of $FASN^{CG17374}$*-RNAi*, but not of $FASN^{CG3524}$*-RNAi*, are significantly different from control. (H) Mating choice tests using single Canton-S females (*F*) [or males (*M*)] in the presence of one Canton-S male (or female) and one RNAi-expressing male (or female). Bars correspond to the percentage of copulations with Canton-S (white bar) or RNAi-expressing flies (gray for *Oe>*$FASN^{CG3524}$*-Ri* and black for *Oe>*$FASN^{CG17374}$*-Ri*). n ⩾ 50. Chi-squared probability tests: from left to right, $P = 0.57$, 0.89, 0.41, and 0.27.

FASN-deficient flies produce few CHCs

To get further insights into the requirement of FA synthesis in CHC biogenesis, we took advantage of a mutant ($FASN^{\Delta 24\text{-}23}$) that removes both $FASN^{CG3524}$ and $FASN^{CG3523}$ genes. This mutant is lethal at the L1 stage but can be rescued by an appropriate lipid-supplemented diet (21). We also induced $FASN^{CG17374}$*-RNAi* with the *1407-gal4* driver in $FASN^{\Delta 24\text{-}23}$ mutants ($FASN^{\Delta 24\text{-}23;CG17374i}$). 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, $FASN^{\Delta 24\text{-}23}$ 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 $FASN^{\Delta 24\text{-}23;CG17374i}$ males (Fig. 3A, A'; supplementary Table 5B), indicating that $FASN^{CG17374}$ can also contribute to the pool of LCFAs used for CHC biogenesis.

To evaluate how these *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 $FASN^{\Delta 24\text{-}23}$ and $FASN^{\Delta 24\text{-}23;CG17374i}$ mutants, TAG stores were dramatically lower at eclosion and almost eliminated 4 days later (Fig. 3B).

Finally, we analyzed *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 *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

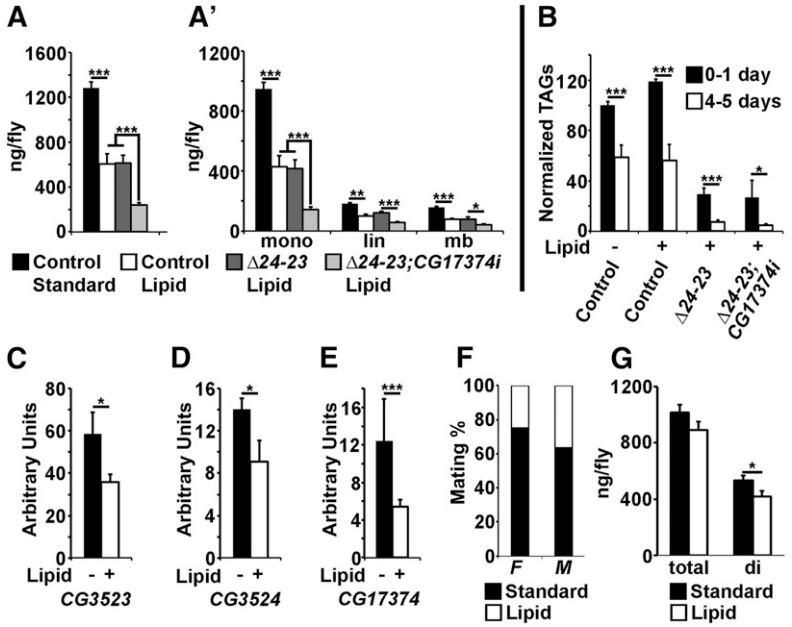

Fig. 3. Lipid feeding and *FASN* mutant analysis. (A) Total CHC amounts in w^{1118} (Control), $FASN^{\Delta 24\text{-}23}$ (Δ*24-23*), and $FASN^{\Delta 24\text{-}23;CG17374i}$ (Δ*24-23;CG17374i*) males emerging from larvae fed either standard (black bar) or lipid-supplemented diet (white, deep gray, and light gray bars). (A') Amounts of monoene (mono), saturated linear (lin), and mb CHC classes in the males represented in A. Statistical analyses are as in Fig. 1C–I. (B) Total TAG levels in w^{1118} (Control), $FASN^{\Delta 24\text{-}23}$ (Δ*24-23*), and $FASN^{\Delta 24\text{-}23;CG17374i}$ (Δ*24-23;CG17374i*) males emerging from larvae fed either standard (-) or lipid-supplemented diet (+). (C–E) Gene expression levels of $FASN^{CG3523}$ (C), $FASN^{CG3524}$ (D), and $FASN^{CG17374}$ (E) in 1-day-old males emerging from larvae fed either standard (black bars) or lipid-supplemented diet (white bars). (F) Mating choice tests of single Canton-S females (left) [or males (right)] in the presence of two w^{1118} males (or females) emerging from larvae fed either standard (black bars) or lipid-supplemented (white bars) diet. n ⩾ 50. Chi-squared probability tests: $P = 0.0004$ (left); $P = 0.04$ (right). (G) Total and diene (di) CHCs in w^{1118} females fed either standard (black bar) or lipid-supplemented diet (white bar).

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 LCFAs 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's 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 $FASN^{CG3523}$, the only *FASN* gene expressed in the FB (19). CHCs were slightly but significantly reduced in *FB>*$FASN^{CG3523}$*-RNAi* flies (Fig. 4I; supplementary Table 6G, H), suggesting that FA synthesis within the FB may participate in feeding the pool of LCFAs 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

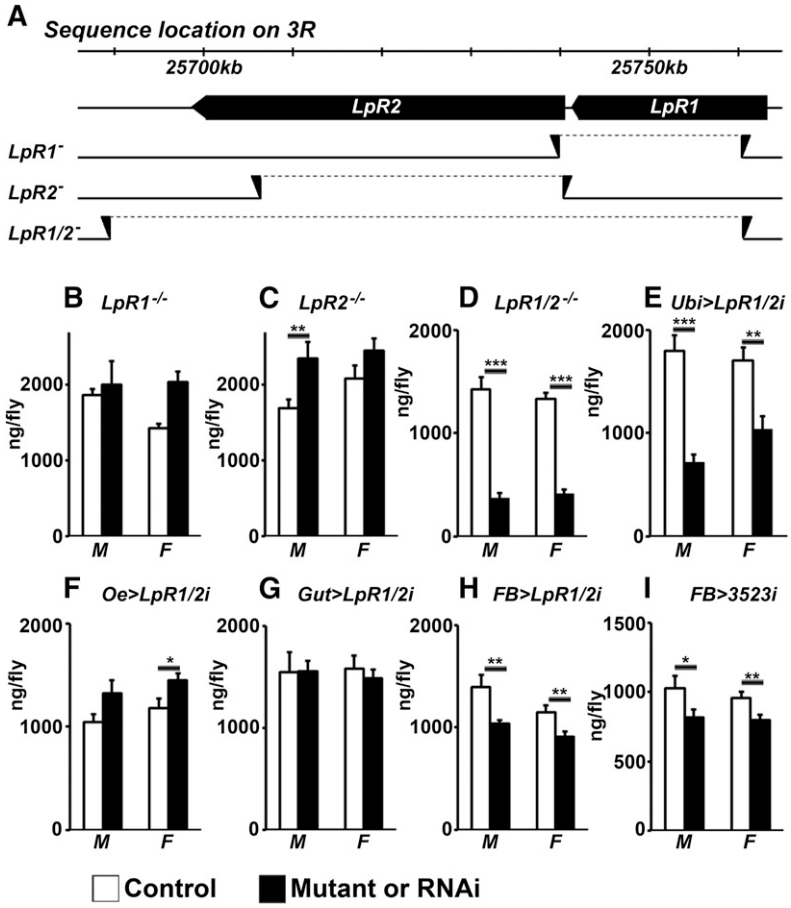

Fig. 4. Role of LpR1, LpR2, and $FASN^{CG3523}$ in CHC biogenesis. A: Scheme of the *LpR1* and *LpR2* deletion mutants and of a deletion that removed both genes (*LpR1/2*). (B–D) Total CHC amounts in male (left) and female (right) *LpR1* (B), *LpR2* (C), or *LpR1/2* (D) deletion mutants (black bars); controls (white bars) are heterozygous mutants. (E–H) Total CHC amounts in males (left) and females (right) expressing *LpR1-RNAi* and *LpR2-RNAi* together with ubiquitous (E), oenocyte (F), gut (G), or FB (H) targeted *gal4* drivers. (I) Total CHC amounts in males (left) and females (right) expressing $FASN^{CG3523}$*-RNAi* with an FB *gal4* driver. Control for RNAi-expressing flies and statistical analyses are as in Fig. 1C–I.

(Fig. 5E). Further, we observed a dramatic accumulation of LDs in *ACC*-deficient oenocytes (Fig. 5F) and to a lesser extent in KAR^{CG1444}-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

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 *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 synthetized within the

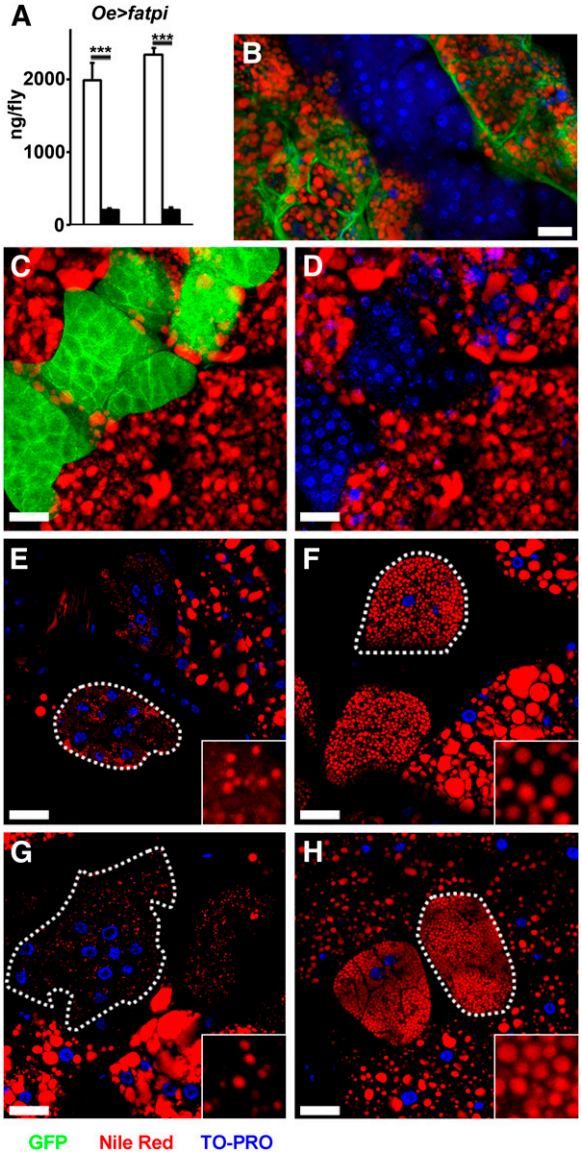

Fig. 5. Function of FATP in the oenocytes for CHC biogenesis. (A) Total CHCs in *Oe>fatp-RNAi* males (left) and females (right). Control and RNAi-expressing flies and statistical analyses are as in Fig. 1C–I. (B–H) Nile Red (red) and nuclei (blue) staining and GFP fluorescence (green in B, C) in abdominal oenocytes and FB of adult flies; expression of GFP in the FB (B) or in the oenocytes (C, D); oenocyte expression of RNAi's to *fatp* (E), *ACC* (F), *KAR* (G), or *fatp* and *ACC* together (H). FB cells are larger than oenocytes and contain large LDs. A group of oenocytes is encircled by a dotted line in E, F, G, and H. Scale bars represent 20 μm.

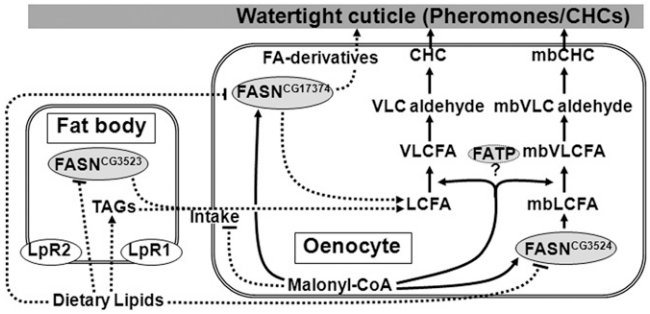

Fig. 6. CHC biogenesis integrates physiological inputs. Bold arrows are enzymatic processes already known or strongly suspected to sustain CHC synthesis in oenocytes. Dotted arrows are enzymatic or regulatory processes highlighted in our study: synthesis of non-CHC compounds required for cuticle watertightness; malonyl-CoA inhibition of lipid uptake; FB lipid metabolism modulating CHC synthesis; role of dietary lipids in feeding the pool of CHC precursors and repressing *FASN* gene expression; and FATP potentially acting as an ACS. Note that lipid intake within the oenocytes may proceed through lipoprotein particles or free FAs.

oenocytes and therefore do not require FA-mediated intake. Nonetheless, we cannot exclude that the FA-transporter domain of FATP is required for CHC biogenesis. However, the increase in LD content induced by ACC knockdown in the oenocytes must be due to lipid intake that does not require FATP. Moreover, as for defaults in VLCFA synthesis, oenocyte knockdown of *fatp* results in almost total CHC depletion, a phenotype that is never observed when LCFA synthesis is impaired, even in a severe *FASN* mutant combination. Consistent with this, biochemical analysis on the strongest mammalian homolog, FATP4, revealed that it acts as an ACS on either LCFA or VLCFA substrates (34). 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 *Drosophila 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, $FASN^{\Delta 24\text{-}23}$ 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 $FASN^{\Delta 24\text{-}23}$ mutant. This suggests either that oenocyte knockdown of $FASN^{CG17374}$ is compensated by increased lipid uptake into the oenocytes or that $FASN^{CG17374}$ is recruited within the oenocytes only when FA synthesis is repressed in the entire animal. In summary, our findings reveal that CHC production integrates various physiological inputs and suggest that regulation of CHC biogenesis operates at the level of LCFA rather than VLCFA synthesis. This regulatory loop integrates nutrition and FB metabolism at the organismal level to guarantee the production of a functional integument. In this process, the FB, which

has been previously shown to control body homeostasis (25, 36), may provide LCFAs to the oenocytes and/or control oenocyte activity.

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