

A Fatty Acid Anabolic Pathway in Specialized-Cells Remotely Controls Oocyte Activation in Drosophila

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22 ABSTRACT

23

24 Pheromone-mediated partner recognition is crucial for maintenance of animal species. Here, we 25 discover a metabolic link between pheromone and gamete physiology. In female genital tract, 26 oocyte maturation is arrested at a specific meiotic-phase. Release of this arrest, called oocyte-27 activation, is triggered by a species-dependent signal. We show in Drosophila melanogaster 28 that oenocytes, which produce the fatty acids (FAs) used as precursors of cuticular 29 hydrocarbons (CHCs), including pheromones, are also essential for oocyte activation. We 30 identified a set of FA-anabolic enzymes required within oenocytes for the synthesis of a 31 particular FA that is not a CHC precursor but controls oocyte activation. Our study thus reveals 32 that two tightly linked FA-anabolic pathways act in parallel, one to produce sexual pheromones, 33 the other to initiate embryonic development. Given that pheromone-deficient Drosophila 34 melanogaster females are highly attractive for males irrespective of their species, this oenocyte 35 function might have evolved to prevent hybrid development.

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44 INTRODUCTION

45 In multicellular organisms, sexual reproduction is crucial for species survival. Meeting mates 46 requires pheromonal signals between individuals of the same species (Rekwot, Ogwu et al., 47 2001). After mating, sperm remains in the female genital tract, where its survival duration varies 48 amongst species (Neubaum & Wolfner, 1999). In mammalian females, sperm entry into the 49 oocyte triggers a calcium signal that induces oocyte activation (Kashir, Nomikos et al., 2014, 50 Miao & Williams, 2012, Swann & Lai, 2016). The regulation of sexual reproduction integrates 51 several physiological inputs, which are not fully characterized. Given the plethora of genetic 52 tools allowing tissue targeted gene miss-expression, Drosophila melanogaster provides a 53 convenient model system to address these issues.

54 In Drosophila females, oogenesis produces oocytes arrested at metaphase I of meiosis. Oocyte 55 activation also depends on a calcium signal (Sartain & Wolfner, 2013), which is not triggered by 56 sperm entry, but proceeds while the oocyte moves through the oviduct and the uterus (Heifetz, 57 Yu et al., 2001). Activation provokes i) meiosis completion, ii) formation of the vitelline 58 membrane that prevents polyspermy and iii) translation of maternally provided mRNAs (Aviles-59 Pagan & Orr-Weaver, 2018, Horner & Wolfner, 2008b, Krauchunas & Wolfner, 2013). In well-fed 60 fertilized females, eggs are continuously produced and spermatozoa stored in the seminal 61 receptacle and spermathecae are synchronously delivered to fertilize the oocytes, indicating 62 that regulatory processes coordinate nutritional status with oogenesis, and egg laying with 63 sperm delivery (Avila, Bloch Qazi et al., 2012, Wolfner, 2011).

Drosophila sexual pheromones are cuticular hydrocarbon (CHC) members (Montagne J. & Wicker-Thomas, 2020) that form by decarboxylation of very long chain fatty acids (VLCFAs) (Qiu, Tittiger et al., 2012). In eukaryotes, VLCFA synthesis is catalyzed by the elongase complex from a LCFA (long chain fatty acid) substrate (Jakobsson, Westerberg et al., 2006), whereas LCFA synthesis is catalyzed by FASN (fatty acid synthase) from an acetyl-CoA primer (Maier, Leibundgut et al., 2010). Synthesis of both LCFAs and VLCFAs requires the sequential incorporation of malonyl-CoA units, whose biogenesis is catalyzed by the Acetyl-CoA

71 carboxylase (ACC) (Barber, Price et al., 2005). Regarding Drosophila CHCs, we previously 72 showed that synthesis of VLCFAs takes place exclusively in the oenocytes, which are rows of 73 cells located underneath the dorsal abdominal cuticle, whereas the LCFAs used as precursors 74 are of flexible origin (Wicker-Thomas, Garrido et al., 2015). The Drosophila genome encodes 75 three FASN genes, two of them, FASN2 and FASN3, are specifically expressed in the 76 oenocytes (Chung, Loehlin et al., 2014, Garrido, Rubin et al., 2015, Parvy, Napal et al., 2012). It 77 has been shown that FASN2 catalyzes the synthesis of methylated/branched(mb)FAs, using a 78 primer distinct from acetyl-CoA (Chung et al., 2014, Wicker-Thomas et al., 2015). Regarding 79 FASN3, we previously reported that its knockdown affects tracheal waterproofing in larvae 80 (Parvy et al., 2012) and desiccation resistance but not CHC synthesis in adult flies (Wicker-81 Thomas et al., 2015). Here, we show that a particular FA-anabolic pathway operating from the 82 oenocytes remotely controls oocyte activation. Our study thus suggests a metabolic link 83 between pheromone synthesis and female fertility.

84

85 **RESULTS**

86 Identification of FA-metabolic genes required in the oenocytes for female fertility

87 While studying CHCs, we observed that Drosophila females deficient for FASN3 in their 88 oenocytes exhibited a sterile phenotype. To identify additional genes potentially involved in this 89 process, we directed inducible interfering RNA (RNAi) to 57 genes encoding enzymes related to 90 FA-metabolism, using the 1407-gal4 driver that is active in oenocytes from mid L3 larval stage 91 to adulthood (Wicker-Thomas et al., 2015). In their progeny, at least twenty virgin females-92 hereafter referred as 1407>geneX-RNAi— were individually crossed to Canton-S males. Next, 93 females were transferred every second day to new vials and the number of emerging adults was 94 counted. This way, we identified four additional gene products required for fertility (Table EV1 95 and Fig EV1A), including ACC, a component of the elongase complex (KAR), a bipartite FA-96 transporter/acyl-CoA ligase (FATP) and CG6432, which encodes a putative short chain acyl-97 CoA ligase. Sibling males of the sterile females were tested for their ability to fertilize Canton-S

females, yet none of them appeared to be sterile (Fig EV1B). Taken together, these findings
reveal that a VLCFA produced in the oenocytes controls female but not male fertility.

100

101 Searching for oenocyte defects

102 KAR, which encodes a component of the elongase complex, has also been shown to prevent 103 oenocyte degeneration in adult flies (Chiang, Tan et al., 2016). We therefore, investigated 104 whether the oenocyte knockdowns that produced female sterility also affected oenocyte viability 105 in females. Consistently, 1407>KAR-RNAi resulted in oenocyte degeneration in 27-day old 106 females, but not in 10- and 18-day old females (Fig EV2A-F). As previously described (Wicker-107 Thomas et al., 2015), 1407>ACC-RNAi induced lipid accumulation in the oenocytes but did not 108 affect their viability (Fig EV2G-I), whereas 1407>FATP-RNAi resulted in oenocyte degeneration 109 that was visible as of day 18 (Fig EV2J-L). In contrast, 1407>FASN3-RNAi and 1407>CG6432-110 RNAi flies were fully viable and the oenocytes of females did not degenerate, did not 111 accumulate high amounts of lipid droplets and appeared similar to controls (Fig EV2M-R). 112 Following knockdown of any of the genes identified, the female sterile phenotype was invariably 113 observed as of day 8, ie. when oenocytes appeared viable (Fig EV2D,G,J,M,P), indicating that 114 the sterile phenotype resulted from the inactivation of a specific FA metabolic pathway, rather 115 than deficient oenocytes.

116

117 Metabolic pathway required for female fertility

118 *CG6432* is proposed to encode a short chain acyl-CoA ligase (Flybase, 2003). However, protein 119 blast analysis revealed that it contains a putative propionyl-CoA synthase domain and that its 120 closest homologue is a short chain FA-acyl-CoA ligase in mouse (Acss3), and an acetyl-CoA 121 ligase in yeast (Acs1) (Fig EV3). Therefore, we investigated whether *CG6432* was also required 122 in other FA-anabolic pathways. We previously reported that oenocyte knockdown of *ACC*, 123 *FASN3, KAR* and *FATP* in young larvae results in flooding of the tracheal system and in lethality

124 at the L2/L3 transition (Parvy et al., 2012). As previously reported for FASN3 (Garrido et al., 125 2015), this phenotype also happened for oenocyte or ubiquitous knockdown of CG6432 (Fig 126 1A), indicating that the CG6432 gene product resides in the oenocyte-specific metabolic 127 pathway that controls tracheal watertightness in larvae. We also previously reported that 128 knockdown of ACC, KAR and FATP, but not of FASN3, in adult oenocytes results in a drop of 129 total CHC amounts (Wicker-Thomas et al., 2015). Knockdown of CG6432 in adult oenocytes did 130 reduce CHC amounts but resulted not total in а dramatic drop of 131 methylated/branched(mb)CHCs (Table EV2, Fig 1B and Fig EV4), a phenotype previously 132 reported in FASN2 oenocyte-knockdown flies (Chung et al., 2014, Wicker-Thomas et al., 2015). 133 In contrast to FASN1, fat body knockdown of CG6432 did not reduce total triacylglycerol levels 134 (Fig 1C), indicating that it is not required for FASN1 activity in the fat body. Taken together, 135 these findings reveal that the enzyme encoded by CG6432 selectively resides in the FASN2 136 and FASN3 but not FASN1 anabolic pathway.

137 Next, we made use of the promE-gal4 driver, which, in contrast to the 1407-gal4 driver (Wicker-138 Thomas et al., 2015), is active only in the oenocytes in Drosophila females as of stage L1 139 (Billeter, Atallah et al., 2009). All the genes required in adult oenocytes for female fertility (Table 140 EV1) are also essential in larval oenocytes for spiracle watertightness, except the elongase subunit elo^{CG6660}, the knockdown of which seemed to affect only the latter process (Parvy et al., 141 2012). Therefore, to retest elo^{CG6660} with the promE-gal4 driver we used a different RNAi line 142 143 (Table EV1). Given that promE-gal4 induced larval lethality when driving any of the genes of 144 interest, its activity was blocked until early metamorphosis using the thermo-sensitive Gal4 145 inhibitor, Gal80^{ts}. RNAi expression was induced by a temperature shift to 27°C. Emerging 146 females were maintained at 27°C, mated 3-5 days after virgin collection and changed to new 147 vials every second day (Fig 1D). In this way, newly fertilized females expressing any of the 148 RNAis of interest exhibited a net reduction of fertility compared to that of control females, which 149 quickly dropped to complete sterility (Fig 1D). Moreover, in contrast to the fertility of 1407>elo^{CG6660}-RNAi females (Fig EV1A), promE-gal4>elo^{CG6660}-RNAi females appeared to be 150

151 sterile, possibly because of different RNAi strength (Fig 1D). Taken together, these findings 152 reveal that a FA anabolic pathway working in the oenocytes produces a particular VLCFA that is 153 required for female fertility (Fig 1E). While the acyl-CoA primer for mbLCFA synthesis catalyzed 154 by FASN2 is likely a propionyl-CoA, whereas the one used by FASN3 is yet unknown. Thus, it is 155 tempting to speculate that CG6432 is responsible for the synthesis of this unconventional FA 156 primer (Fig 1E).

157

158 Oenocytes control female fertility

159 To get further insights into this oenocyte function, we focused on FASN3 and CG6432, since 160 both these genes are essential only in the oenocytes and their knockdown alters neither 161 oenocyte viability (Fig EV2) nor CHC synthesis (Fig 1B and (Wicker-Thomas et al., 2015)). 162 Furthermore, confocal imaging of oenocytes (Fig 2A) and ovaries (Fig 2B) of promE>FASN3-163 RNAi and promE>CG6432-RNAi females dissected 10 days after mating, revealed no apparent 164 defects, all the egg chamber stages were visible (Fig 2B). Moreover, in mating assays, single 165 wild type males did not exhibit any significant preference when given a choice between a wild 166 type female and a female of either genotype promE>FASN3-RNAi or promE>CG6432-RNAi 167 (Fig 2C). Next, we monitored the number of eggs laid by females. Control, promE>FASN3-RNAi 168 and promE>CG6432-RNAi females laid high amounts of eggs the day after fertilization (Fig 2D); 169 this number decreased the following days, although this effect was more pronounced for 170 promE>FASN3-RNAi and promE>CG6432-RNAi compared to control females (Fig 2D). 171 However, consistent with a fertility defect, the number of eggs that formed pupae in the FASN3-172 and CG6432-RNAi conditions dropped more dramatically than the number of eggs laid, as 173 compared to control (Fig 2E-F). Taken together, these findings reveal that the oenocyte 174 metabolic pathway that controls female fertility affects neither partner mating nor oogenesis.

175

176 **Oenocytes do not control sperm delivery to the oocytes**

177 After copulation, spermatozoa are stored for several days within the seminal receptacle and 178 spermathecae of Drosophila females (Wolfner, 2011). We searched for potential defects in 179 sperm storage or delivery. In control females, the number of spermatozoa in the seminal 180 receptacle progressively decreased after mating, while females laid eggs continuously (Fig 3A); 181 promE>FASN3-RNAi and promE>CG6432-RNAi females contained roughly the same number 182 of spermatozoa in their seminal receptacle the day after mating, but surprisingly, this number decreased much less during the following days compared to control females (Fig 3A). Next, we 183 184 monitored sperm motility in the seminal receptacle but did not observe a difference in sperm 185 speed in promE>FASN3-RNAi and promE>CG6432-RNAi females compared to control females 186 (Fig 3B and movies EV1-3). Moreover, confocal analysis revealed that the eggs of control, 187 promE>FASN3-RNAi and promE>CG6432-RNAi females were fertilized as shown by the 188 presence of sperm flagella (Fig 3C-E). These findings indicate that, despite sperm retention in 189 storage organs, the defect of fertility is not due to a failure of sperm entry into the oocyte.

190

Oenocytes control oocyte activation

192 A fertilized egg contains potentially five nuclei, the sperm and oocyte pronuclei, plus three polar 193 globules. However, while analyzing the presence of spermatozoa, we noticed an apparent 194 defect in the number of nuclei in promE>FASN3-RNAi and promE>CG6432-RNAi females (Fig 195 3C-E). We therefore, counted the number of nuclei in eggs laid by virgin females and observed 196 that the number of nuclei in control eggs varid from zero to four, likely because some nuclei are 197 not stained or not visible (Fig 4A and 4D). Nevertheless, the number of nuclei in 198 promE>FASN3-RNAi and promE>CG6432-RNAi females was much lower than in control 199 females (Fig 4B-C and 4D). Production of the three polar globules results from meiosis 200 completion (Page & Orr-Weaver, 1997), suggesting that this process does not fully operate in 201 promE>FASN3-RNAi and promE>CG6432-RNAi females. Meiosis completion is triggered by 202 oocyte activation, which also induces formation of the vitelline membrane and translation of 203 maternally provided mRNAs (Horner & Wolfner, 2008a). The vitelline membrane allows

204 resistance to bleach induced egg lysis (Horner, Czank et al., 2006). Importantly, we observed 205 that fertilized eggs laid by promE>FASN3-RNAi and promE>CG6432-RNAi females were much 206 less resistant to bleach treatment (Fig 4E). However, the bleach concentration required for egg 207 lysis was higher than the one described by others (Horner et al., 2006). Finally, we analyzed 208 Smaug, a protein encoded by a maternally provided mRNA, whose translation is induced by egg 209 activation (Horner & Wolfner, 2008a, Tadros, Goldman et al., 2007). Western-blot analysis 210 revealed that Smaug was present at much lower levels in eggs laid by promE>FASN3-RNAi 211 and promE>CG6432-RNAi than in eggs laid by control females (Fig 4F). Taken together these 212 findings reveal that a particular VLCFA produced in the oenocytes controls oocyte activation.

213

214 **DISCUSSION**

215 In this study, we provide evidence that a FA-anabolic pathway, which takes place within the 216 oenocytes of Drosophila females, remotely controls fertility. We have identified six FA-anabolic enzymes required for this process: ACC, FASN3, CG6432, FATP, KAR and Elo^{CG6660}. ACC, the 217 218 rate-limiting enzyme for FA synthesis, catalyzes malonyl-CoA synthesis (Barber et al., 2005). 219 FASN3 is one of the three Drosophila FASN enzymes (Garrido et al., 2015). CG6432 that 220 encodes a putative short chain acyl-CoA synthase, might catalyze the synthesis of the acyl-CoA 221 primer used by FASN2 and FASN3 to build up FAs (Fig 1D). FATP is a bipartite fatty acid 222 transporter/acyl-CoA synthase, although our previous study supports that it works instead as an 223 acyl-CoA synthase tightly linked to VLCFA synthesis (Wicker-Thomas et al., 2015). KAR and Elo^{CG6660} are subunits of the elongase complex that comprises two reductases (KAR and TER), 224 225 a dehydratase and the elongase (Jakobsson et al., 2006). The elongase subunit assigns 226 specificity to FA primer usage and to the VLCFA produced; both of which remain unidentified for Elo^{CG6660}, as for most of the 20 elongases encoded by the *Drosophila* genome (Montagne J. & 227 228 Wicker-Thomas, 2020). Nonetheless, the oenocyte-mediated control of female fertility likely 229 relies on either a single or a restricted subclass of VLCFA(s), which might be the precursor of a 230 yet unknown lipid hormone. We also observed that oenocyte knockdown of FATP and KAR, but 231 not of ACC, FASN3 and CG6432, results in oenocyte degeneration, further supporting the 232 notion that FATP operates closely to VLCFA synthesis (Wicker-Thomas et al., 2015). 233 Potentially, the degeneration process is not induced by the lack of VLCFAs but by the 234 accumulation of LCFA precursors, which happens when inhibiting VLCFA but not LCFA 235 synthesis. These six enzymes are also required in larval oenocytes to maintain the spiracle 236 watertightness (Parvy et al., 2012), suggesting that FASN3-dependent VLCFAs are the 237 precursors of lipid messengers transmitted through the haemolymph to their target tissues. Our 238 study demonstrates that one of these particular VLCFAs operates on oocyte activation. In 239 females deficient for this VLCFA, i) the low number of nuclei in unfertilized eggs suggests that 240 meiosis completion does not fully operate, ii) the bleach sensitivity of eggs argues for a vitelline 241 membrane defect, and iii) the low levels of Smg suggests a defect in the translation of 242 maternally provided mRNAs. All these processes are typically triggered by oocyte activation 243 (Aviles-Pagan & Orr-Weaver, 2018). Nonetheless, the lack of this remote control is unlikely to 244 result in a total blockade of oocyte activation, since all these processes are affected but not fully 245 suppressed.

246 At first glance, it is surprising that a VLCFA synthesized through a metabolic pathway tightly 247 parallel to the one responsible for pheromone biogenesis, remotely controls oocyte activation. 248 Of note, it has been shown that Drosophila melanogaster females devoid of oenocytes are 249 attractive to males from other species, including D. simulans, D. yakuba and D. erecta (Billeter 250 et al., 2009). These females are also more attractive to wild type Drosophila melanogaster 251 males, since the delay for copulation with oenocyte deficient females in shortened. The 252 increased attractiveness likely depends on carboxyl-methylated FAs produced by Drosophila 253 females, since shortening of the copulation delay does not happen with males deficient for the 254 odorant receptor of these carboxyl-methylated FAs. These studies support the notion that 255 female attractiveness depends on a balance between repulsion and attraction, where the 256 cocktail of CHC-related compounds would be rather repulsive, while the species distinctive 257 pheromone signature confers the selective attractiveness for conspecific males. Given that

258 pheromone biogenesis shares common enzymes with the synthesis of the oocyte-activating 259 VLCFA, deficiency in the former pathway should also impede the latter. Therefore, it is tempting 260 to speculate that these two pathways have been co-selected throughout evolution to prevent 261 hybrid development. Flies harboring non-functional oenocytes would copulate with males 262 irrespective of their species with the resulting hybrids being potential competitors. Identification of the FASN3-dependent VLFCAs will constitute the next challenge and should allow 263 264 deciphering its action mechanism on oocyte activation and determining whether a similar 265 regulatory process is conserved throughout evolution to favor species isolation.

266

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273

Author contributions: CWT and JM conceived the study; MP, NM, PD, BD, MD, DP, CWT and JM performed the methodology; MP, GW, CWT and JM analyzed the data; JM wrote the manuscript. All authors reviewed the manuscript.

277

278 **Competing interest**: The authors declare that they have no conflict of interest.

279

280 MATERIAL AND METHODS

281 Genetics and fly handling

282 Fly stock: 1407-gal4 (oenocytes from mid L3 stage) (Ferveur, Savarit et al., 1997), BO-gal4 283 (oenocytes in embryo and early larvae) (Gutierrez, Wiggins et al., 2007), promE-gal4 284 (oenocytes from L1 stage) (Billeter et al., 2009), P[w8, ProtB-DsRed-monomer, w+]50A UAS-285 GFP (Manier, Belote et al., 2010), Cg-gal4 (fat body), da-gal4 (ubiquitous), Tub-gal80ts 286 (ubiquitous) from BDSC (https://bdsc.indiana.edu). Inducible UAS-RNAi lines (Table S1) from 287 VDRC (https://stockcenter.vdrc.at/control/main) (Dietzl. Chen NIG et al.. 2007). 288 (https://shigen.nig.ac.jp/fly/nigfly) or previously described (Palm, Sampaio et al., 2012, Parvy et 289 al., 2012)

290

291 Mating and related analyses

292 Mating choice were performed as previously described (Wicker-Thomas et al., 2015). For sperm 293 counting and speed, females were fertilized with Plw8. ProtB-DsRed, w+1 males that labelled 294 sperm nuclei. For counting, spermathecae and seminal receptacle were dissected in PBS 1X, 295 fixed 20 min in PFA 4%, mounted in DABCO and spermatozoa were counted with a Zeiss 296 Imager M2 fluorescent microscope. For sperm speed, seminal receptacle were dissected and 297 mounted in Biggers, Whitten and Whittingham modified medium (95 mM NaCl, 4.8 mM KCl, 1.3 298 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 5.6 mM glucose, 25 mM NaHCO3, 20mM 299 HEPES, 0.6% fatty acid free BSA, pH 7.6), supplemented with 0.5 mM trehalose. Time lapse 300 were imaged with Zeiss Imager M2 fluorescent microscope (10 seconds; 0.15 second frame 301 interval); speed means are from at least 20 spermatozoa per genotype (five females each). For 302 bleach resistance, eggs were collected for 2 hrs, incubated with commercial bleach (3,7% 303 chlorax) for 10 mn, rinsed with water, and numbers of eggs still visible were counted.

304

305 Imaging

306 Dissected oenocytes and ovaries were fixed and labeled with DAPI and Oil-Red-O as previously 307 described (Wicker-Thomas et al., 2015). For sperm flagella immunostaining, eggs were

dechorionated for 2 min in commercial bleach (3,7% chlorax), fixed in a 1:1 heptane:methanol mixture and stored at -20°C. Next, embryos were washed three times for 10 min with PBS 0.1% Triton X100, incubated with primary antibody (anti-acetylated tubulin; Sigma-Aldrich; T6793) and DAPI on a rotating wheel overnight at 4°C, washed three times (20 min each) and incubated with an anti-mouse antibody Alexa Fluor 568 nm (Invitrogen; A11061). Samples were mounted in DAPCO and analyzed on a Leica SP8 confocal laser-scanning microscope.

314

315 **Biochemistry**

Experiments performed as previously described: CHC measurement (Wicker-Thomas et al., 2015). TAG measurement (Garrido et al., 2015); protein extracts and western-blotting (Montagne, Lecerf et al., 2010). The Smaug antibody was kindly provided by M Simonelig (Chartier, Klein et al., 2015). Quantification of western-blot was performed using ImageJ.

320

321 Statistics

322 Statistical analysis were performed using PRISM/Graphpad. Statistical significance were 323 indicated as *, ** and *** corresponding to P< 0.05, 0.01 and 0.001, respectively. T-test were 324 used for Fig. 1B, 1C, 1D, 2D, 2E, 3A, 3B, 4E, 4F. Chi-2 test were used for Fig. 2C and 4D. 325 ANOVA was used for Table S2.

326

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416 **Figure legends**

417 Figure 1: Characterization of an oenocyte FA anabolic pathway required for female 418 fertility: (A) Oenocyte(BO)- or ubiquitous(da)-knockdown of CG6432 induced tracheal flooding 419 in late L2 larvae; in their flooded section, the tracheal trunks (arrows in control: Co) were hardly 420 visible. (B) CHCs amounts in control (black) or 1407>6432-RNAi (green) females (n=10); 421 Amounts of dienes (di), monoenes (mono), saturated linear (sat), pheromones (HD+ND) and 422 mbCHCs (mb) are listed in Table EV2; note the strong reduction of mbCHCs. (C) TAG content 423 in 0-5 hours prepupae of the following genotypes: Cg-gal4 control (black), Cg>FASN3-RNAi 424 (purple), Cg>CG6432-RNAi (green) and Cg>FASN1-RNAi (grey). (D) Pupal progeny of promE-425 gal4 females either control (black) or expressing an RNAi to FASN3 (purple), KAR (orange), CG6432 (green), FATP (dark grey), ACC (light grey) or elo^{CG6660} (white). Five females were 426 mated with five males for two days (0-2d), then, males were removed and females transferred in 427 428 new tubes every second day (2-4d, 4-6d, 6-8d). Each bar represents the man value of 3-5 429 replicates. (E) Oenocyte anabolic pathway producing a VLCFA controlling fertility, where 430 CG6432 and FATP are potential acyl-CoA synthase for the primer used by FASN2/FASN3 and 431 for FA elongation, respectively; enzymes (blue/red) and metabolites (black) are indicated.

432 Figure 2: Oogenesis and mating: (A-B) Oenocytes (yellow dotted line in A1-3) and stage 9-10 433 (*) and late (#) egg chambers (B1-3) of promE-gal4 females either control (A1, B1), or directing 434 an RNAi to FASN3 (A2, B2) or CG6432 (A3, B3); tissues were dissected 10-days after mating; 435 lipids and nuclei were labelled with Nile Red (red) and DAPI (silver), respectively; scale bars: 40 436 µm (A1-3) and 100 µm (B1-3). (C) Mating choice of single wild type males in the presence of 437 two females, one control and one expressing an RNAi to either FASN3 (left) or CG6432 (right); 438 bars represent the percentage of copulation with control (color) or RNAi-expressing (purple or 439 green) females; males tend to prefer CG6432-RNAi females although not significantly. (D-F) 440 eggs (D) and pupae (E) from promE-gal4 females either control (black) or expressing an RNAi 441 to FASN3 (purple) or CG6432 (green); three females were mated with three males for one day, 442 then, males were removed and females transferred in new tubes every day over a 6-day period; 443 index of fertility (F) were evaluated as the ratio of prepupae to eggs.

444 Figure 3: Sperm activity: (A) Sperm numbers in the spermathecae (left) and seminal 445 receptacle (right) of promE-gal4 females either control (Co) or directing an RNAi to FASN3 or 446 CG6432, one day (black) or five days (grey) after mating (n>25). (B) Movement speed of 447 spermatozoa in the seminal receptacle of promE-gal4 females either control (black), or directing 448 an RNAi to FASN3 (purple) or CG6432 (green). (C-E) Eggs laid from promE-gal4 fertilized 449 females either control (C), or directing an RNAi to FASN3 (D) or CG6432 (E); eggs were 450 collected for 40 mn, nuclei were labeled with DAPI (silver) and sperm flagella (arrows) with an 451 anti-acetylated-tubulin (red); scale bars: 40 µm.

Figure 4; Oocyte activation: (A-C) Eggs laid from *promE-gal4* virgin females either control (A), or directing an RNAi to *FASN3* (B) or *CG6432* (C); eggs were collected for 2 hrs and nuclei were labeled with DAPI (silver); scale bars: 40 μm. (D) Number of nuclei counted in the egg collections (A-C). (E) Resistance to bleach lysis of eggs laid by *promE-gal4* fertilized females either control (black), or directing and RNAi to *FASN3* (purple) or *CG6432* (green); bars represent means from 27 independent tests, each containing 25 eggs per genotype. (F) Western-blotting to Smaug from protein extracts of dissected ovaries or eggs laid by *promE-*

- 459 gal4 females either virgin or fertilized; ovaries and unfertilized eggs were from control females,
- 460 fertilized eggs were from females either control or directing an RNAi to *FASN3* or *CG6432*. The
- 461 graph at the bottom compares the means of four independent blots, where the band intensity of
- 462 Smaug was normalized to that of the tubulin loading control.

463









Figure 3





A Fatty Acid Anabolic Pathway in Specialized-Cells Remotely Controls Oocyte Activation in *Drosophila*

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EXTENDED VIEW

Figure EV1. Screening for sterility.

Figure EV2. Oenocytes structure during lifespan.

Figure EV3. CG6432 blast.

Figure EV4. male CHCs.

Table EV1. List of the genes screened for female sterility

Table EV2. Statistical and CHC detailed analyses

Movie EV1. Displacement of spermatozoa in the seminal receptacle of promE-gal4 control females.

Movie EV2. Displacement of spermatozoa in the seminal receptacle of *promE-gal4*>FASN3-Ri.

Movie EV3. Displacement of spermatozoa in the seminal receptacle of promE-gal4>CG6432-Ri.



Figure EV1: Screening for sterility. (**A**) *1407-gal4>UAS-RNAi* females crossed to Canto-S males were let to lay eggs during six days (D) in three successive vials and the progeny was counted at adult emergence (N). (**B**) Reciprocal crosses to test male fertility.





Figure EV2: Oenocytes structure during lifespan. Dorsal abdominal cuticles stained for lipids and nuclei of *1407>UAS-GFP* females either control (A-C), or directing UAS-RNAis to KAR (D-F), ACC (G-I), FATP (J-L), FASN3 (M-O) or CG6432 (P-R). Females were dissected 9 days (A,D,G,J,M,P), 18 days (B,E,H,K,N,Q) or 27 days (C,F,O,R) after adult emergence, except ACC- and FATP-RNAis flies that did not survive longer than 24 days (I,L). Oenocytes were visualized by GFP (green) the nuclei by DAPI (silver) and the fat body by Nile red. Since the Nile red partially interferes with the GFP channel and that the GFP intensity varied a lot for unknown reasons, the lipid staining appeared either red (strong GFP) or orange (low GFP). Note that oenocyte loss appeared earlier in age for FATP-RNAi (K,L) than for KAR-RNAi (F). Scale bars: 100µm.

13 Dm MKPSWLQCRKVTGAGTLGAP-----LPGSPSVRGAAVTRRALVAGF 41 Mm MSPSAVQSSKLEEQSSEIDKLKAKMSQSAATAQQKKEHEYEHLTSVK--IVPQRPISDRL 58 Sc -ESV--CGMPVEAHDPLYLKAYRQSVQNPAAFWEEQG-NLLDWDRPWEKVLDNSN-----64 Dm GGRG--CRALTTGSGGEYKTHFAASVADPERFWGKAA-EQISWYKPWTKTLESRY----93 Мm QPAIATHYSPHLDGLQDYQRLHKESIEDPAKFFGSKATQFLNWSKPFDKVFIPDPKTGRP 118 Sc . *: :* *: . . : :.* :*: *.: * -PPFTKWYVGGYLNACYNSIDRHILAGRGSNVALIHDSPLTGTLRRVTYQELYDQIVLLA 123 Dm 152 Mm -PPSTSWFVEGMLNICYNAIDRHIENGQGDKIAIIYDSPVTDTKATISYKEVLEQVSKLA SFQNNAWFLNGQLNACYNCVDRHALKTPN-KKAIIFEGDEPGQGYSITYKELLEEVCQVA 177 Sc . *:: * ** ***.:*** . : *:*.:. . ::*:*: ::: :* GGLA-KLGVVKGDRVVIYMPLIPETIIAMLAIVRLGAIHSVVFGGFAARELCSRIEHVEP Dm 182 Mm GVLV-KQGVKKGDTVVIYMPMIPQAIYTMLACARIGAIHSLIFGGFASKELSTRIDHAKP 211 SC QVLTYSMGVRKGDTVAVYMPMVPEAIITLLAISRIGAIHSVVFAGFSSNSLRDRINDGDS 237 *. . ** *** *.:***::*: :** *:****::*.***:..* **:. . KLVIASNVGVEPGKVVPYLDILHSAISMSRWRPPQRNIIFRRDNVSPDTTKLDPLTDVLW 242 Dm KVVVTASFGIEPGRKVEYIPLLEEALRIGQHRPDR-VLIYSRPNMEK--VPLMSGRDLDW 268 Mm KVVITTDESNRGGKVIETKRIVDDALRETP--GVRHVLVYRKTNNPS--VAFHAPRDLDW 293 Sc *:*::: . . *: : ::..*: : :::: * . : *: * SDILKMAEGERPIACVPIEANDPLYILYTSGTTDKPKGVLRTIGGHLVALVYTLRTLYGI 302 Dm 325 EEEMAK---AQSHDCVPVLSEHPLYILYTSGTTGLPKGVVRPTGGYAVMLNWTMSSIYGL Мm ATEKKKY--KTYYPCTPVDSEDPLFLLYTSGSTGAPKGVQHSTAGYLLGALLTMRYTFDT 351 SC *.*: ::.**::****:*. **** : .*: : *: 362 NPGHTWWAASDMGWVVGHSYICYGPLCLGATSVMYEGKPDRTPDPGQYFRIIDQYQVRSI Dm 385 KPGEVWWAASDLGWVVGHSYICYGPLLHGNTTVLYEGKPVGTPDAGAYFRVLAEHGVAAL Mm 410 HQEDVFFTAGDIGWITGHTYVVYGPLLYGCATLVFEGTPA-YPNYSRYWDIIDEHKVTQF Sc *: . *: :: :: * FSVPTSFRVIRRADPDISYGRQYSMKSLRAIFIAGEHCDYETKSWIEKT---FKVPVLNH 419 Dm FTAPTAIRAIRQQDPGAALGKQYSLTRFKTLFVAGERCDVETLEWSKKV---FRVPVLDH 442 Mm YVAPTALRLLKRAG--DSYIENHSLKSLRCLGSVGEPIAAEVWEWYSEKIGKNEIPIVDT 468 SC : .**::* ::: . : .::*:. :: : .** *. .* .: .:*::: WWQTETGSAVTATCLGFQQNLSPPTYSTGLPLMGYDVKILKADGSEAQ-TSEL-GRIALK 477 Dm WWQTETGSPITASCIGLGNSKTPPPGQAGKCVPGYNVMILDDNMQKLK-ARSL-GNIVVK 500 Mm YWQTESGSHLVTPLAGGVT--PMKPGSASFPFFGIDAVVLDPNTGEELNTSHAEGVLAVK 526 Sc :***:** :.: .:. . * :. :*. : : * : * :.:* LPLPPGNMATLYKNEELFRTLYFQKFPGYYDTMDAGYKDERGYIFVTARDDDVINVAGHR 537 Dm 560 Мm LPLPPGAFSGLWKNQEAFKHLYFEKFPGYYDTMDAGYMDEEGYLYVMSRVDDVINVAGHR Sc A-AWPSFARTIWKNHDRYLDTYLNPYPGYYFTGDGAAKDKDGYIWILGRVDDVVNVSGHR 585 *:: :**** * *.. *: **::: .* ***:** *. ::**.: : 591 LSTSSLEDAVLRHPDVVDVAVFGVPEATKGQVPLCLYIPVENCKKTD-----AKLSTEI Dm ISAGAIEESVLSHGTVADCAVVGKEDPLKGHVPLALCVLKKDVNASE-----EQVLEEI 614 Мm LSTAEIEAAIIEDPIVAECAVVGFNDDLTGQAVAAFVVLKNKSSWSTATDDELQDIKKHL 645 SC :*:. :* ::: . *.: **.* : .*:. .: : :... .: .: 649 Dm IKLIRDVVGPIAAFRLVTSVNNLPRTRSGKTMRKAMADFARNERVVLP--ATIDDASVFI Mm VKHVRQSIGPVAAFRNAVFVKQLPKTRSGKIPRSTLSALVNGKPYKVT--PTIEDPSIFG 672 Sc VFTVRKDIGPFAAPKLIILVDDLPKTRSGKIMRRILRKILAGESDQLGDVSTLSNPGIVR 705 * : : .: *:.: .:. : :*. :**.** : *.:**:**** : Dm EIRRALNQLGYAMTAPDPIVAKLLD 674 HIEEVLKOAV-----682 Мm HLIDSVKL-----713 Sc :: . :

Figure EV3: CG6432 blast. Peptide sequence alignment of CG6432 (Dm) to the best homologues Acss3 in mouse (Mm) and Acs1 in yeast (Sc), using <u>www.uniprot.org</u>.



Figure EV4: male CHCs. Means values of CHCs from 10 control (white) or 10 *CG6432-RNAi* (black) males.

CG	Name, Expected function	Line	Stock	Fertility
1444	Ketoacyl-DH/VLCFA synthesis	40949	VDRC	Sterile
2781	Elongase, VLCFA synthesis	102543	VDRC	+
3523	FASN1, LCFA synthesis	29349	VDRC	+
3524	FASN2, LCFA synthesis	4290	VDRC	+
3961	LCFA-CoA ligase	37305	VDRC	+
3971	Baldspot, Elongase, VLCFA synthesis	47519	VDRC	+
4020	FA-CoA reductase	107095	VDRC	+
4389	Mtpα, b-oxidation	21845	VDRC	+
4501	bgm, LCFA-CoA ligase	105635	VDRC	+
4600	yip2, Thiolase, b-oxidation	26562	VDRC	+
5278	sit, Elongase, VLCFA synthesis	43091	VDRC	+
5326	Elongase, VLCFA synthesis	47681	VDRC	+
5887	desat1, desaturase	104350	VDRC	+
5925	desat2, desaturase	103666	VDRC	+
6432	short chain acyl-CoA ligase	43451	VDRC	Sterile
6660	Elongase, VLCFA synthesis	101046	VDRC	+
6660	Elongase, VLCFA synthesis	6660R-2	NIG	ND
6730	Cyp4d21, monooxygenase	102401	VDRC	+
6921	bond, Elongase, VLCFA synthesis	102051	VDRC	+
7461	Acyl-CoA dehydrogenase, b-oxidation	7461R-4	NIG	+
7910	FA amide hydrolase	51546	VDRC	+
7920	Acetyl-CoA hydrolase	21577	VDRC	+
7923	Fad2, desatF, desaturase		СМТ	+
8522	SREBP, Transcription factor	37640	VDRC	+
8534	Elongase, VLCFA synthesis	107515	VDRC	+
9057	Lsd-2, Lipid storage	40734	VDRC	+
9342	Mtp, Lipid transport	110414	VDRC	+
9390	AcCoAS, Acetate-CoA ligase	100281	VDRC	+
9458	Elongase, VLCFA synthesis	48702	VDRC	+
9459	Elongase, VLCFA synthesis	48905	VDRC	+
9743	FA desaturase	108185	VDRC	+
9747	FA desaturase	1394	VDRC	+
9914	3-hvdroxvacvI-CoA dehvdrogenase	106649	VDRC	+
10374	Lsd-1, Lipid storage	30844	VDRC	+
11064	apolpp, Lipid transport	100944	VDRC	+
11198	ACC, malonyl-CoA synthesis	8105	VDRC	Sterile
11801	Elo688. Elongase. VLCFA synthesis	103506	VDRC	+
12086	cue. LDL receptor	104645	VDRC	+
15531	FA desaturase	1397	VDRC	+
15828	Apolto, Lipid transport		S Eaton	+
16904	Elongase, VLCFA synthesis	106515	VDRC	+
16905	eloF VI CFA synthesis	16905-R1	NIG	+
17374	FASN3 LCFA synthesis	10000 111	JM	Sterile
17560	FA-CoA reductase	104756	VDRC	+
17562	FA-CoA reductase	37365	VDRC	+
17646	ABC transporter-like	100378	VDRC	+
17821	Flongase VI CEA synthesis	/005/0	VDPC	+
18031	FarO FA-CoA reductase	30220	VDRC	+
18600	Flongaso VI CEA synthesis	1001	VDRC	
30009	Flongase VI CFA synthesis	4334	VDPC	- -
311/1	Flongase VI CFA synthesis	100/60	VDPC	+
31522	Flongase VI CFA synthesis	106652	VDPC	- -
31522	Flongase VICEA synthesis	100032	VDPC	
31023	Liongase, VLOFA Synthesis	40220		
32072	Elouu, Elongase, VLOFA synthesis	5200	VDRC	
32919	Faily acyl-COA reductase	6090	VDRC	+
33110	Elongase, VLCFA synthesis	6926-R2	NIG	+
42611	Ingl, LDL receptor	1050/1	VDRC	+ 04
46149	ratp, lipid transport, FA-COA ligase	9406	VDRC	Sterile

Table EV1. List of the genes screened for female sterility (fertility column) using the *1407-gal4* driver. RNAi lines were provided by NIG or VDRC; three of them have been generated in S Eaton, CWT or JM laboratories (Stock column). *UAS-RNAi* lines that produced a sterile phenotype were reused with the *promE-gal4* driver, except 6660R-2 used to knockdown *elo*^{CG6660}.

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Males CHC	Р	6432i-C	6432i
Tot	0.24	1634.1 ± 91.8	1840.3 ± 146.8
9-T	0.003	2.50 ± 0.25	3.78 ± 0.28
7-T	0.002	$51.33 \pm 1.05.2$	55.7 ± 0.61
5-T	0.25	3.72 ± 0.18	4.14 ± 0.31
23:0	0.004	13.54 ± 0.41	14.69 ± 0.30
Me-24	<.001	3.03 ± 0.24	0.38 ± 0.06
9-P	0.003	1.27 ± 0.14	2.24 ± 0.14
7-P	<.001	5.62 ± 0.19	10.24 ± 0.53
5-P	0.13	0.12 ± 0.07	0.34 ± 0.11
25 :0	0.50	1.85 ± 0.19	2.05 ± 0.22
Me-26	<.001	6.68 ± 0.43	0.42 ± 0.11
27:0	<.001	1.20 ± 0.15	2.37 ± 0.21
Me-28	<.001	7.76 ± 0.61	0.62 ± 0.10
29 :0	<.001	0.47 ± 0.11	2.66 ± 0.24

Females CHC	Р	6432i-C	6432i
Tot 23-29	0.26	1291.6 ± 64.7	1574.7 ± 236.1
9-T	0.25	0.70 ± 0.11	0.89 ± 0.11
7-T	0.75	3.32 ± 0.38	3.47 ± 0.29
5-T	0.03	$0.26.{\pm}~0.06$	0.60 ± 0.13
23:0	0.05	11.22 ± 0.62	13.39 ± 0.84
7,11-PD	0.04	2.44 ± 0.30	1.68 ± 0.16
Me-24	<.001	1.88 ± 0.23	0.09 ± 0.01
9-P	1	3.14 ± 0.31	3.14 ± 0.37
7-P	0.96	2.07 ± 0.18	2.06 ± 0.19
5-P	0.42	0.16 ± 0.06	0.23 ± 0.06
25 :0	0.29	5.24 ± 0.28	5.83 ± 0.48
7,11-HD	<.001	18.84 ± 0.72	13.84 ± 0.56
Me-26	<.001	16.37 ± 0.57	0.60 ± 0.14
9-Н	0.05	3.12 ± 0.14	2.56 ± 0.23
7 - H	0.01	2.20 ± 0.16	2.90 ± 0.18
5-H	0.01	0.05 ± 0.02	0.19 ± 0.05
27:0	<.001	2.78 ± 0.23	10.17 ± 0.44
7,11-ND	<.001	14.82 ± 0.52	28.95 ± 1.55
Me-28	<.001	9.23 ± 0.42	1.37 ± 0.34
9-N	0.11	0.19 ± 0.07	0.40 ± 0.10
7-N	0.06	0.22 ± 0.08	0.44 ± 0.08
29 :0	<.001	0.65 ± 0.07	6.50 ± 0.55

Table EV2. Statistical and CHC detailed analyses: Analysis of *1407>CG6432-Ri* (*6432i*) 4-5-day old males (top) or females (bottom). First column: CHC identities; elemental composition is indicated as the carbon chain length followed by the number of double bonds; Me- are mbCHCs. CHCs are expressed in ng/ fly (Tot) or in percentages relative to total CHC amount as the mean (± SEM) of CHCs produced by 10 flies kept for 4 days at 25°C.