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1 **A Fatty Acid Anabolic Pathway in Specialized-Cells Remotely**

2 **Controls Oocyte Activation in *Drosophila***

3
4 Mickael Poidevin^a, Nicolas Mazuras^{b,2}, Gwénaëlle Bontonou^{b,3}, Pierre Delamotte^a, Béatrice
5 Denis^b, Maëlle Devilliers^a, Delphine Petit^a, Claude Wicker-Thomas^{b,1}, Jacques Montagne^{a,1}

6
7 ^a Institut for Integrative Biology of the Cell (I2BC), UMR 9198, CNRS, Université Paris-Sud,
8 CEA, 91190, Gif-sur-Yvette, France

9 ^b Laboratoire Evolution, Génomes, Comportements, Ecologie (EGCE), UMR 9191, CNRS, IRD,
10 Université Paris-Sud and Université Paris-Saclay, 91190, Gif-sur-Yvette, France

11
12 ¹ CWT and JM contributed equally to the work. Correspondence:

13 cwickerthomas@gmail.com ; Jacques.MONTAGNE@i2bc.paris-saclay.fr

14
15 ² Present address: IEES PARIS, UMR 7618, UPEC, 94010 Créteil Cedex, France

16 ³ Present address: Department of Ecology and Evolution, UNIL, CH-1015 Lausanne

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19 **Short title: Oenocytes Control Fertility**

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21

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).

64 *Drosophila* sexual pheromones are cuticular hydrocarbon (CHC) members (Montagne J. &
65 Wicker-Thomas, 2020) that form by decarboxylation of very long chain fatty acids (VLCFAs)
66 (Qiu, Tittiger et al., 2012). In eukaryotes, VLCFA synthesis is catalyzed by the elongase
67 complex from a LCFA (long chain fatty acid) substrate (Jakobsson, Westerberg et al., 2006),
68 whereas LCFA synthesis is catalyzed by FASN (fatty acid synthase) from an acetyl-CoA primer
69 (Maier, Leibundgut et al., 2010). Synthesis of both LCFAs and VLCFAs requires the sequential
70 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

98 females, yet none of them appeared to be sterile (Fig EV1B). Taken together, these findings
99 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 not reduce total CHC amounts but resulted in a 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
141 subunit *elo*^{CG6660}, the knockdown of which seemed to affect only the latter process (Parvy et al.,
142 2012). Therefore, to retest *elo*^{CG6660} with the *promE-gal4* driver we used a different RNAi line
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
150 *1407>elo*^{CG6660}-RNAi females (Fig EV1A), *promE-gal4>elo*^{CG6660}-RNAi females appeared to be

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
183 decreased much less during the following days compared to control females (Fig 3A). Next, we
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

191 **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 varied 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
217 enzymes required for this process: ACC, FASN3, CG6432, FATP, KAR and Elo^{CG6660}. ACC, the
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
224 Elo^{CG6660} are subunits of the elongase complex that comprises two reductases (KAR and TER),
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
227 Elo^{CG6660}, as for most of the 20 elongases encoded by the *Drosophila* genome (Montagne J. &
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 is 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
263 of the FASN3-dependent VLFCAs will constitute the next challenge and should allow
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

274 **Author contributions:** CWT and JM conceived the study; MP, NM, PD, BD, MD, DP, CWT and
275 JM performed the methodology; MP, GW, CWT and JM analyzed the data; JM wrote the
276 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 et al., 2007), NIG
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 *P[w8, ProtB-DsRed, w+]* 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

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

314

315 **Biochemistry**

316 Experiments performed as previously described: CHC measurement (Wicker-Thomas et al.,
317 2015). TAG measurement (Garrido et al., 2015); protein extracts and western-blotting
318 (Montagne, Lecerf et al., 2010). The Smaug antibody was kindly provided by M Simonelig
319 (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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415

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),
426 *CG6432* (green), *FATP* (dark grey), *ACC* (light grey) or *elo^{CG6660}* (white). Five females were
427 mated with five males for two days (0-2d), then, males were removed and females transferred in
428 new tubes every second day (2-4d, 4-6d, 6-8d). Each bar represents the mean 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 .

452 **Figure 4; Oocyte activation: (A-C)** Eggs laid from *promE-gal4* virgin females either control (A),
453 or directing an RNAi to *FASN3* (B) or *CG6432* (C); eggs were collected for 2 hrs and nuclei
454 were labeled with DAPI (silver); scale bars: 40 μm . **(D)** Number of nuclei counted in the egg
455 collections (A-C). **(E)** Resistance to bleach lysis of eggs laid by *promE-gal4* fertilized females
456 either control (black), or directing and RNAi to *FASN3* (purple) or *CG6432* (green); bars
457 represent means from 27 independent tests, each containing 25 eggs per genotype. **(F)**
458 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

464

Figure 1

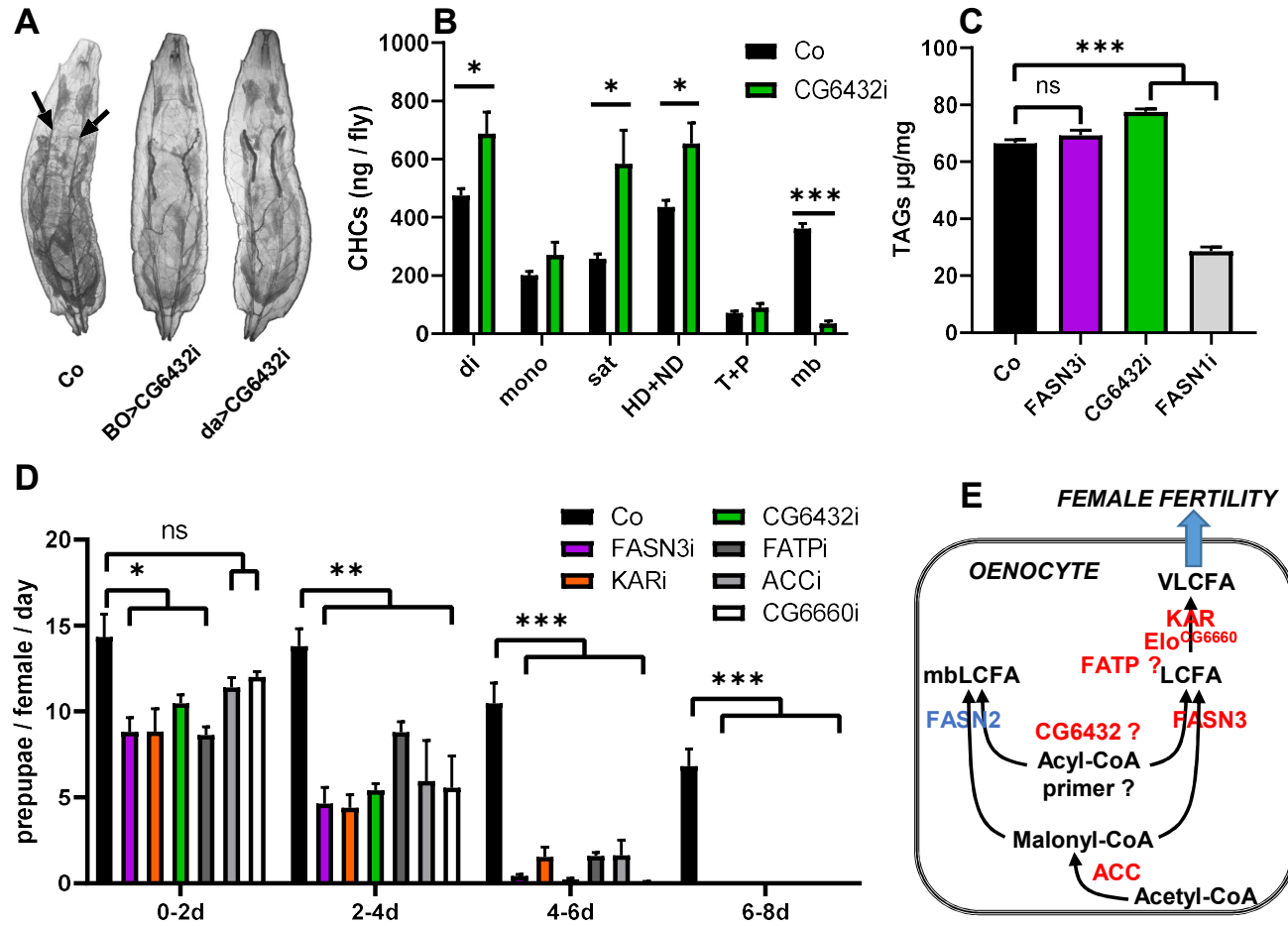


Figure 2

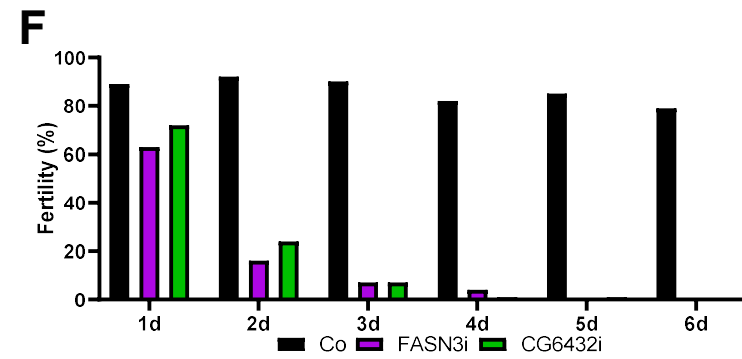
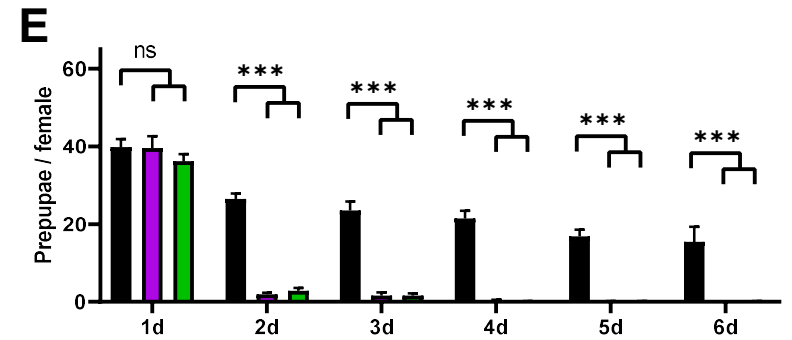
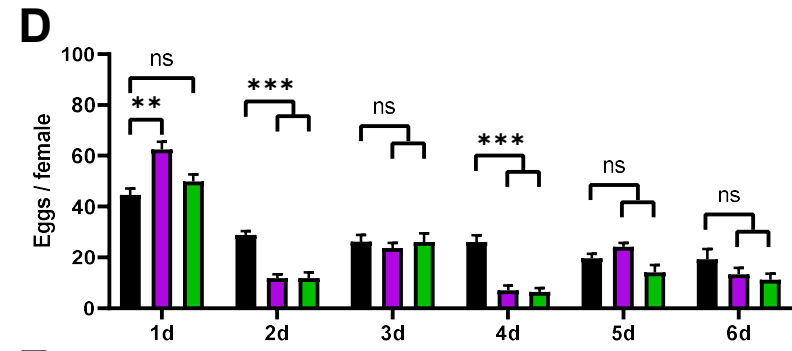
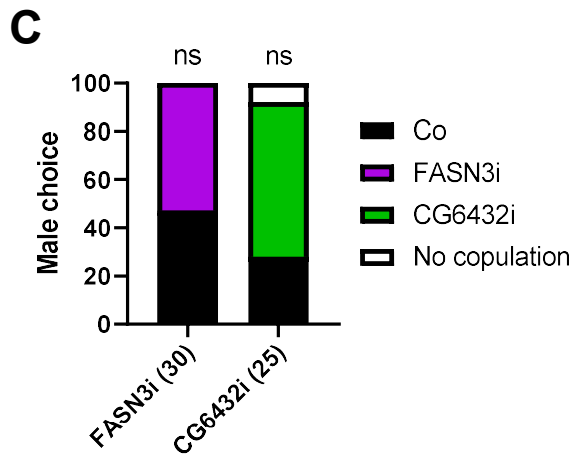
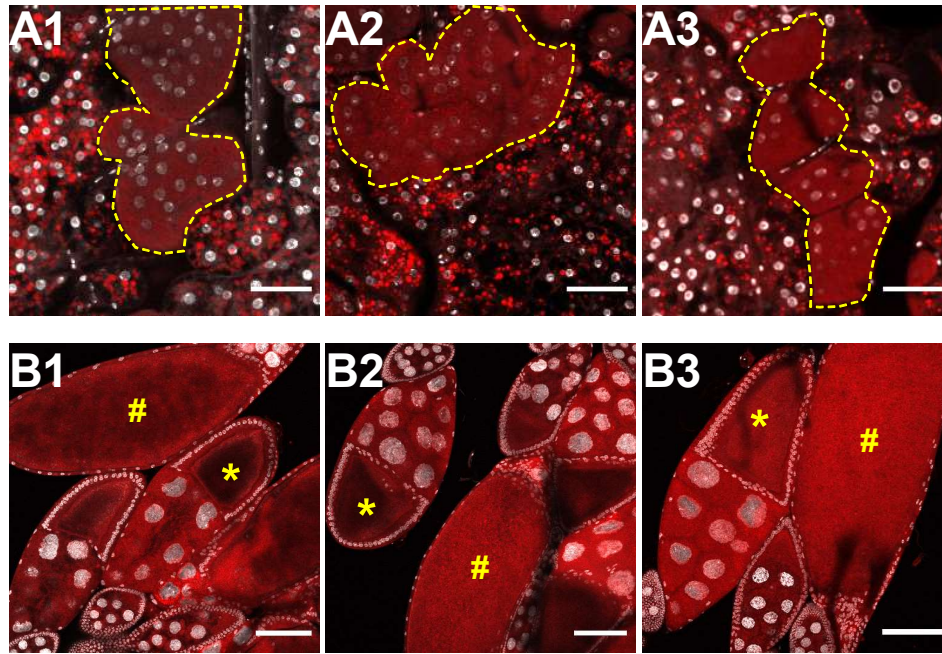


Figure 3

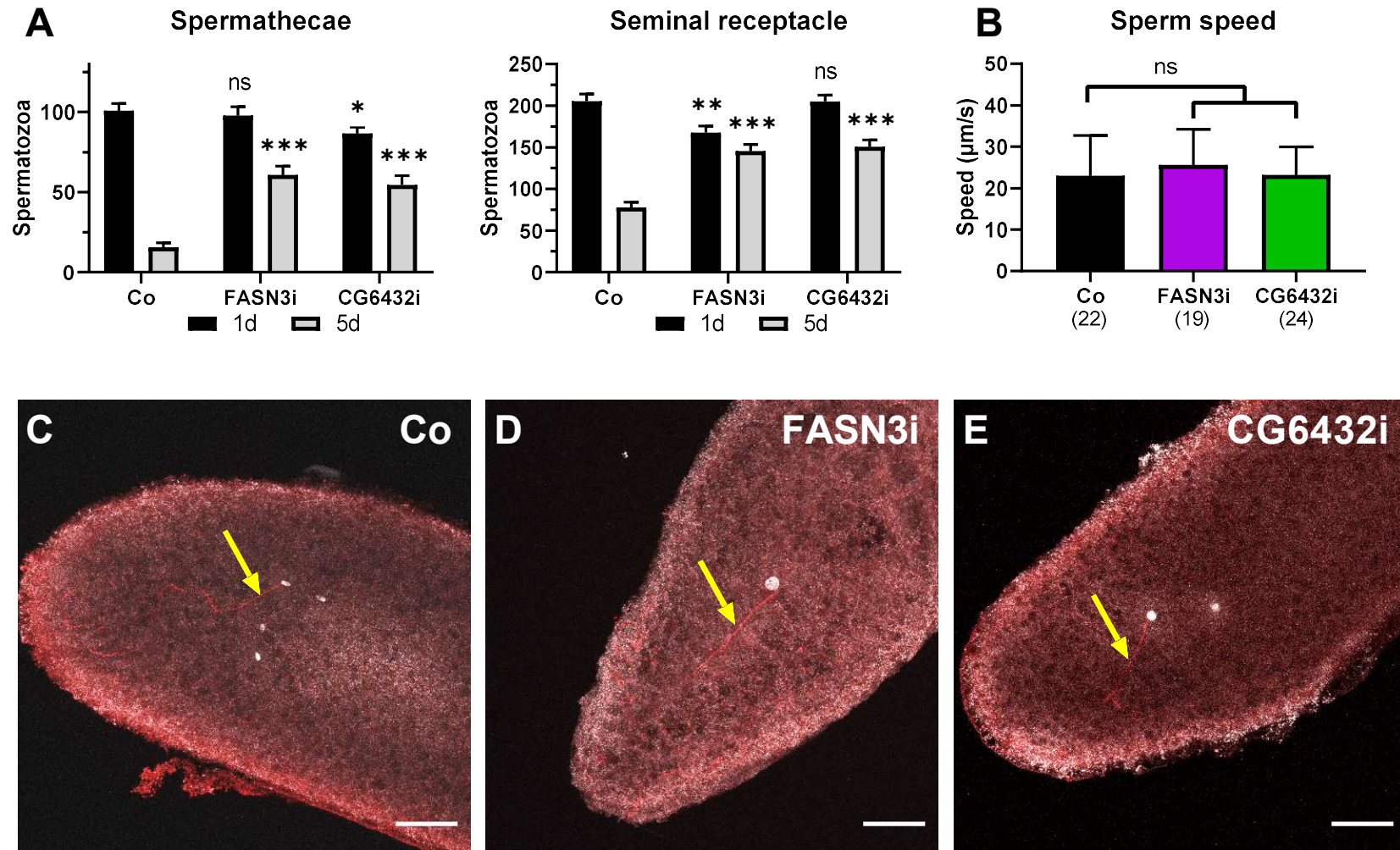
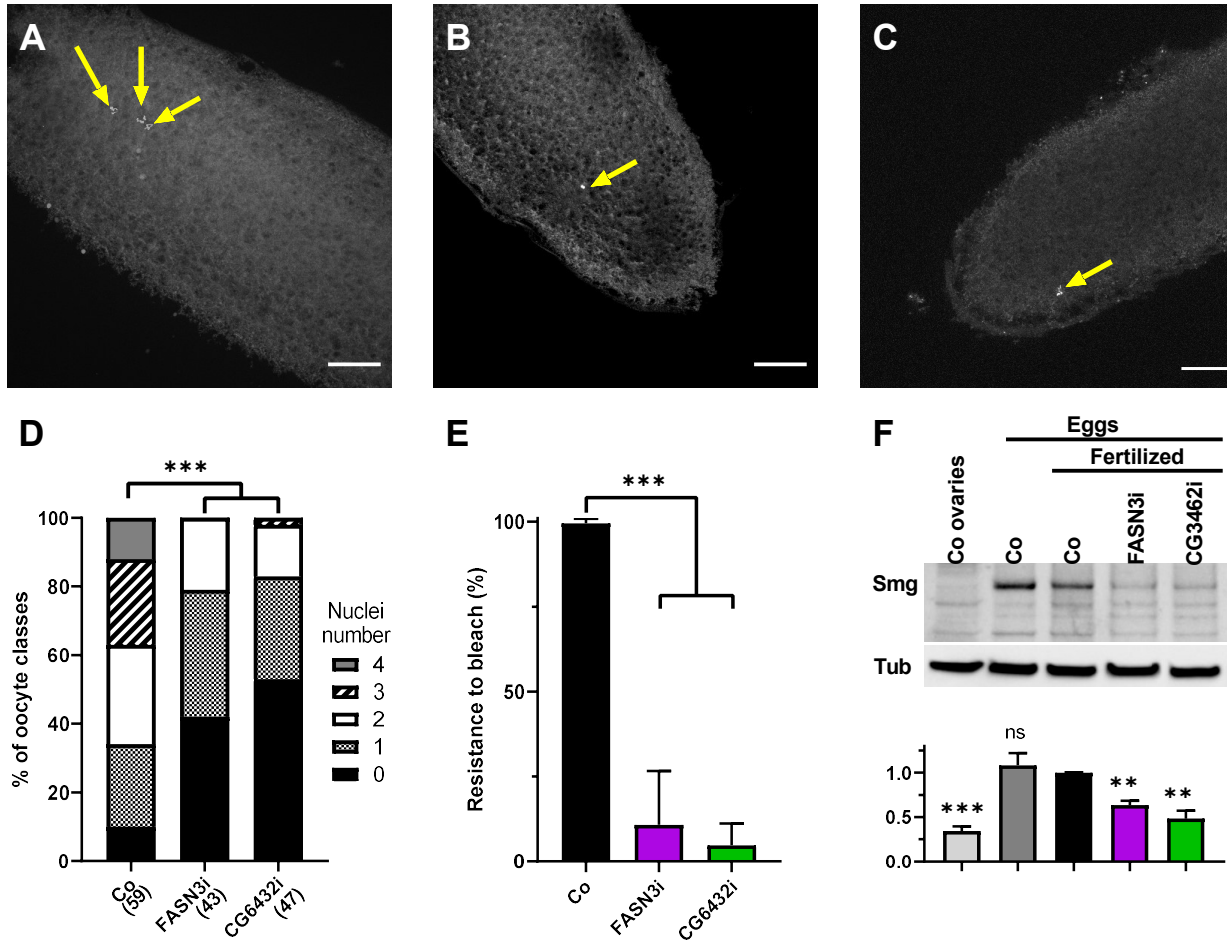


Figure 4



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

Mickael Poidevin, Nicolas Mazuras, Gwénaëlle Bontonou, Pierre Delamotte, Béatrice Denis, Maëlle Devilliers, Delphine Petit, Claude Wicker-Thomas, and Jacques Montagne

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

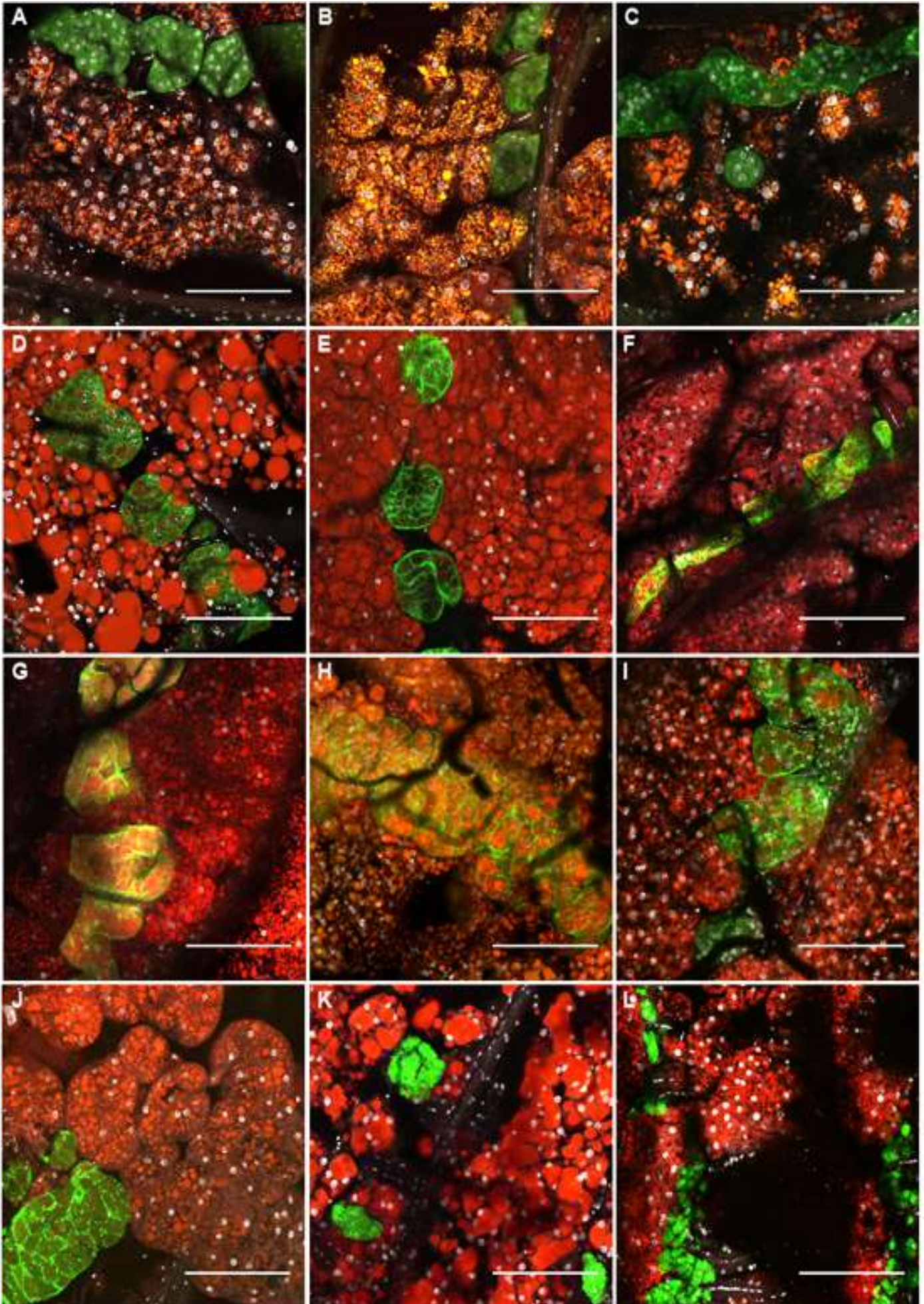
Movie EV1. Displacement of spermatozoa in the seminal receptacle of *promE-gal4* control females.

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.



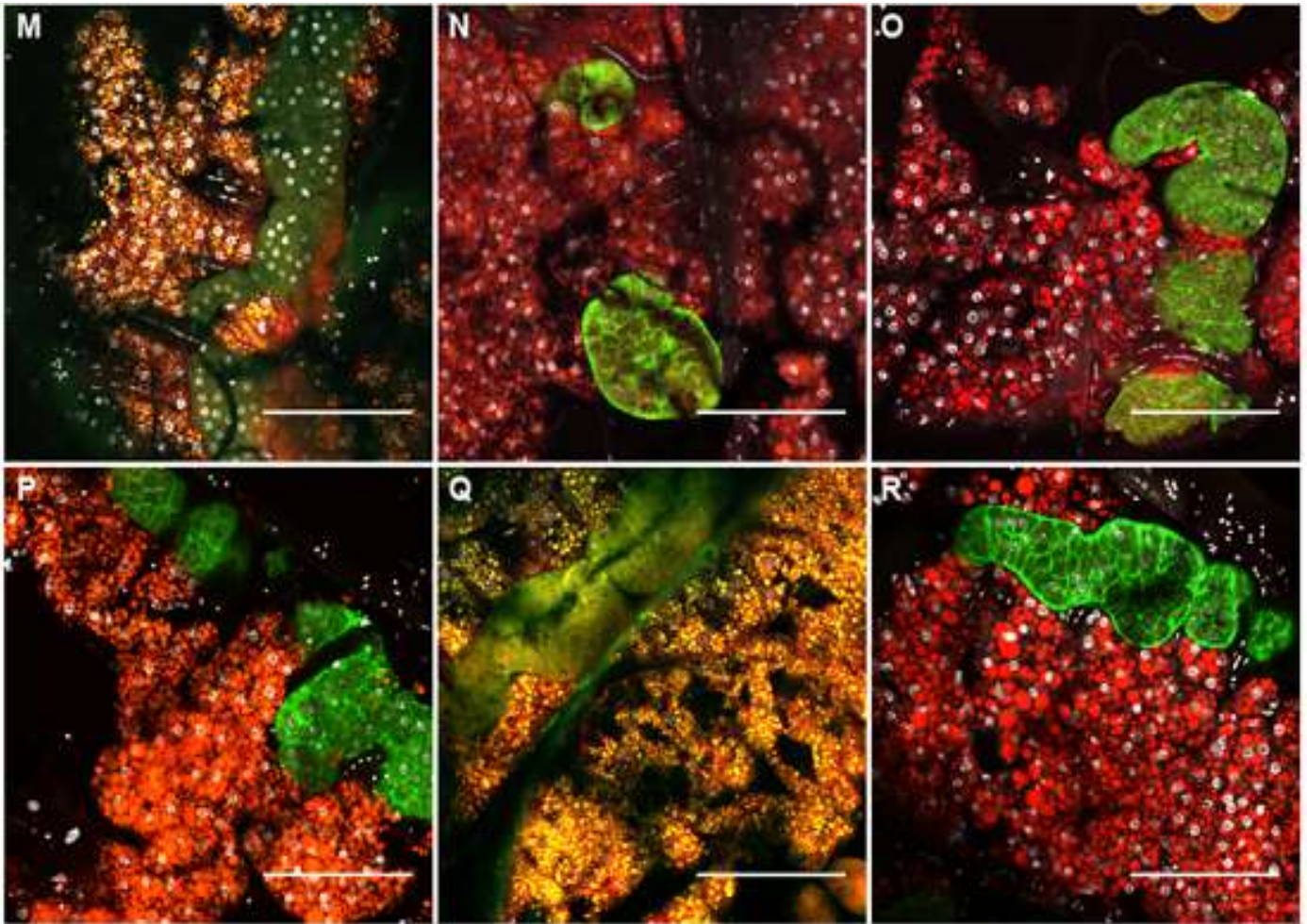


Figure EV2: Oenocytes structure during lifespan. Dorsal abdominal cuticles stained for lipids and nuclei of *1407>UAS-GFP* females either control (A-C), or directing UAS-RNAis to KAR (D-F), ACC (G-I), FATP (J-L), FASN3 (M-O) or CG6432 (P-R). Females were dissected 9 days (A,D,G,J,M,P), 18 days (B,E,H,K,N,Q) 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.

Dm	-----MEPGPSAVN-----YEGS-----	13
Mm	MKPSWLQCRKVTGAGTLGAP-----LPGSPSVRGA AVTRRALVAGF	41
Sc	MSPSAVQSSKLEEQSSEIDK LKAKMSQSAATAQQKKEHEYEHLTSVK--IVPQRPI SDRL	58
.		
Dm	-ESV--CGMPVEAHDPLYLKAYRQSVQNPAAFWEEQG-NLLDWD RPWEKVL DNSN-----	64
Mm	GGRG--CRALTTGSGGEYKTHFAASVADPERFWGKAA-EQISWYKPWTKTLESRY-----	93
Sc	QPAIATHYSPHLDGLQDYQRLHKESIEDPAKFFGSKATQFLNWSKPF DKVFI PDPKTGRP	118
	* . *: :* *: . . : :.* *: *: *	
Dm	-PPFTKWYVGGYL NACYN SIDRH ILAGRGSNVALIHDSPLTGT LRRV TYQELYDQIVLLA	123
Mm	-PPSTSWFVEGMLNICYNAIDRHIENGQGDKIAI IYDSPVTDTKATISYKEVLEQVSKLA	152
Sc	SFQNNAWFLNGQLNACYN CVDRHALKTPN-KKAI I FEGDEPGQGSITYKELLEEV CQVA	177
	. *: : * ** ***. : *** . : * : * . . . : * : * : : : *	
Dm	GGLA-KLGVVKGDRVVIYMP LI PETI IAMLAI VRLGAIHSVVF GGFAARELCSRIE HVEP	182
Mm	GVLV-KQGVKKGDTVVIYMPMI PQAIY TMLACARIGAIHSLIFGGFASKELSTRIDHAKP	211
Sc	QVLTYSM GVRKGD TVAVYMPMPEAIITLLAISRIGAIHSVVFAGFSSNSLRDRINDGDS	237
	* . . ** ** * . : *** : : * : * * : * : * : * : * : * : * : *	
Dm	KLVIASNVGVEPGKVVPYLDILHSAISMSRWRPPQRNIIFRRDNVSPDTTKLDPLTDVLW	242
Mm	KVVVTASFGIEPGRKVEYIPLLEEALRIGQHRPDR-VLIYSRPNMEK--VPLMSGRDL DW	268
Sc	KVVIITDESNRGGKVIETKRIVDDALRETP--GVRHVLVYRKTN NPS--VAFHAPRDL DW	293
	* : * : : . . * : : : : . * : : : : * : : : * : *	
Dm	SDILKMAEGERPIACVPIEANDPLYI LY TSGTTDKPKGVLRTIGGHLVALVYTLR TLTYGI	302
Mm	EEEMAK---AQSHDCVPVLSEHPLYI LY TSGTTGLPKGVVRPTGGYAVMLNWTMSS IYGL	325
Sc	ATEKKKY--KTYYPCTPV DSEDP LFLLYTSGSTGAPKGVQHSTAGYLLGALLTMR YTFDT	351
	* . * : : . * : : * : * : * : * : * : * : * : * : *	
Dm	NPGHTWWAASDMGWVVGHSYICYGPLCLGATSVMYEGKPDRTPDPGQYFRIIDQYQVRSI	362
Mm	KPGEVWWAASDLGWVVGHSYICYGPLLHGNTTVLYEGKPVGTPDAGAYFRVLAEHGVAAL	385
Sc	HQEDVFFTAGDIGWITGHTYVVYGPLLYGCATLVFEGTPA-YPNYSRYWDI IDEHKVTQF	410
	: . . : : * . * : * : * : * : * : * : * : * : * : * : *	
Dm	FSVPTSFRVIRRADPDISYGRQYSMKSLRAIFIAGEHCDYETKSWIEKT---FKVPVLNH	419
Mm	FTAPTAIRAIRQDDPGAALGKQYSLTRFKTLFVAGERCDVETLEWSKKV---FRVPVL DH	442
Sc	YVAPTALRLLK RAG--DSYIENHSLKSLRCLG SVGEPIAAEVW EWYSEKIGKNEIPIVDT	468
	: . * : * : * : : . : : * : * . : * : * : * : * : *	
Dm	WWQTETGSAVTATCLGFQQLNSPPTYSTGLPLMGYDVKILKADGSEAQ-TSEL-GRIALK	477
Mm	WWQTETGSPITASCIGLGN SKT PPPGQAGKCVPGYNMILDDNMQK LK-ARSL-GNIVVK	500
Sc	YWQTESGSHLVTPLAGGVT--PMKPGSASF PFFGIDAVVLD PNTGEELNTSHAEGVLAVK	526
	: * * : * * : : . * . . * : * : * : * : * : * : *	
Dm	LPLPPGNMATLYKNEELFRTLYFQKFPGYD TMDAGYKDERGYIFVTARDDVINVAGHR	537
Mm	LPLPPGAFSGLWKNQEA FKHLYFEKFPGYD TMDAGYMDEEGYLYVMSRVDDVINVAGHR	560
Sc	A-AWPSFARTIWKNHDRYLD TYLNPYPGY YFTGDGAAKDKDGYIWILGRVDDVNVSGHR	585
	* . : * * : : * : : * * * * * * . * : * * : : . * * * : * * * *	
Dm	LSTSSLEDAVLRHPDVVDVAVFGVPEATKGQVPLCLYIPVENCKKTD-----AKLSTEI	591
Mm	ISAGAIEESVLSHGTVADCAVVGKEDPLKGHVPLALCVLKKD VNASE-----EQVLEEI	614
Sc	LSTAEIEAAI IEDPIVAECAVVGFNDDLTGQAVAAFVVLKNKSSWSTATDDELQDIKKHL	645
	: * : . * : : . * : * * * : . * : . : : : . . : : . : *	
Dm	IKLIRDVVGPIAAFR LVTSVNNLPRTRSGK TMRKAMADFARNERVVLP--ATIDDASVFI	649
Mm	VKHVRQSIGPVAAFRNAV FVKQLPKTRSGKIPRSTLSALVNGKPYKVT--PTIEDPSIFG	672
Sc	VFTVRKDIGPFAAPKLIILVDDL PKTRSGKIMRRILRKILAGESDQLGDVSTLSNPGIVR	705
	: * . : * * * * : * : * * : * * * * * * : : . : : * : * : . .	
Dm	EIRRALNQLGYAMTAPDPIVAKLLD	674
Mm	HIEEVLKQAV-----	682
Sc	HLIDSVKL-----	713
	. : . :	

Figure EV3: CG6432 blast. Peptide sequence alignment of CG6432 (Dm) to the best homologues Acsc3 in mouse (Mm) and Acs1 in yeast (Sc), using www.uniprot.org.

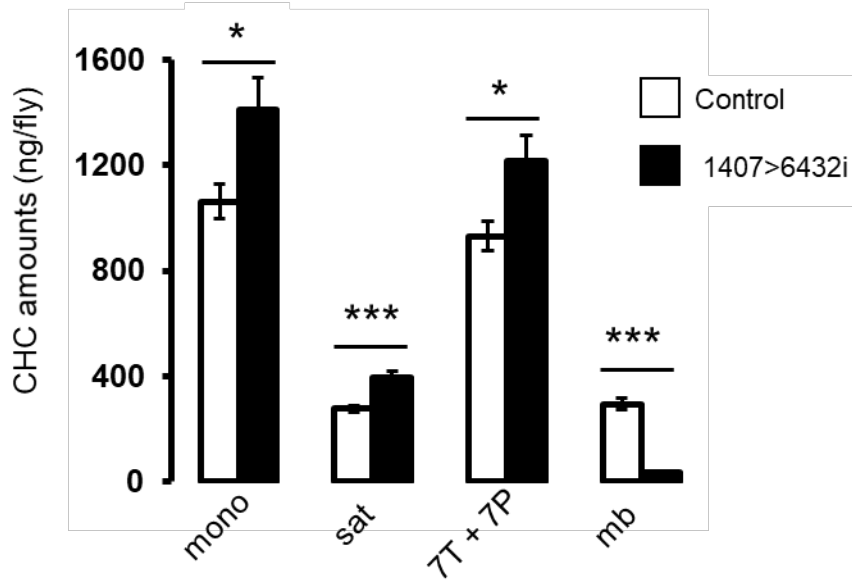


Figure EV4: male CHCs. Means values of CHCs from 10 control (white) or 10 *CG6432-RNAi* (black) males.

CG	Name, Expected function	Line	Stock	Fertility
1444	Ketoacyl-DH/VLCFA synthesis	40949	VDRRC	Sterile
2781	Elongase, VLCFA synthesis	102543	VDRRC	+
3523	FASN1, LCFA synthesis	29349	VDRRC	+
3524	FASN2, LCFA synthesis	4290	VDRRC	+
3961	LCFA-CoA ligase	37305	VDRRC	+
3971	Baldspot, Elongase, VLCFA synthesis	47519	VDRRC	+
4020	FA-CoA reductase	107095	VDRRC	+
4389	Mtpα, b-oxidation	21845	VDRRC	+
4501	bgm, LCFA-CoA ligase	105635	VDRRC	+
4600	yip2, Thiolase, b-oxidation	26562	VDRRC	+
5278	sit, Elongase, VLCFA synthesis	43091	VDRRC	+
5326	Elongase, VLCFA synthesis	47681	VDRRC	+
5887	desat1, desaturase	104350	VDRRC	+
5925	desat2, desaturase	103666	VDRRC	+
6432	short chain acyl-CoA ligase	43451	VDRRC	Sterile
6660	Elongase, VLCFA synthesis	101046	VDRRC	+
6660	Elongase, VLCFA synthesis	6660R-2	NIG	ND
6730	Cyp4d21, monooxygenase	102401	VDRRC	+
6921	bond, Elongase, VLCFA synthesis	102051	VDRRC	+
7461	Acyl-CoA dehydrogenase, b-oxidation	7461R-4	NIG	+
7910	FA amide hydrolase	51546	VDRRC	+
7920	Acetyl-CoA hydrolase	21577	VDRRC	+
7923	Fad2, desatF, desaturase		CWT	+
8522	SREBP, Transcription factor	37640	VDRRC	+
8534	Elongase, VLCFA synthesis	107515	VDRRC	+
9057	Lsd-2, Lipid storage	40734	VDRRC	+
9342	Mtp, Lipid transport	110414	VDRRC	+
9390	AcCoAS, Acetate-CoA ligase	100281	VDRRC	+
9458	Elongase, VLCFA synthesis	48702	VDRRC	+
9459	Elongase, VLCFA synthesis	48905	VDRRC	+
9743	FA desaturase	108185	VDRRC	+
9747	FA desaturase	1394	VDRRC	+
9914	3-hydroxyacyl-CoA dehydrogenase	106649	VDRRC	+
10374	Lsd-1, Lipid storage	30844	VDRRC	+
11064	apolpp, Lipid transport	100944	VDRRC	+
11198	ACC, malonyl-CoA synthesis	8105	VDRRC	Sterile
11801	Elo68β, Elongase, VLCFA synthesis	103506	VDRRC	+
12086	cue, LDL receptor	104645	VDRRC	+
15531	FA desaturase	1397	VDRRC	+
15828	Apoltp, Lipid transport		S Eaton	+
16904	Elongase, VLCFA synthesis	106515	VDRRC	+
16905	eloF, VLCFA synthesis	16905-R1	NIG	+
17374	FASN3, LCFA synthesis		JM	Sterile
17560	FA-CoA reductase	104756	VDRRC	+
17562	FA-CoA reductase	37365	VDRRC	+
17646	ABC transporter-like	100378	VDRRC	+
17821	Elongase, VLCFA synthesis	4997	VDRRC	+
18031	FarO, FA-CoA reductase	30220	VDRRC	+
18609	Elongase, VLCFA synthesis	4994	VDRRC	+
30008	Elongase, VLCFA synthesis	6760	VDRRC	+
31141	Elongase, VLCFA synthesis	100460	VDRRC	+
31522	Elongase, VLCFA synthesis	106652	VDRRC	+
31523	Elongase, VLCFA synthesis	45226	VDRRC	+
32072	elo68α, Elongase, VLCFA synthesis	9206	VDRRC	+
32919	Fatty acyl-CoA reductase	6090	VDRRC	+
33110	Elongase, VLCFA synthesis	6926-R2	NIG	+
42611	mgl, LDL receptor	105071	VDRRC	+
46149	Fatp, lipid transport, FA-CoA ligase	9406	VDRRC	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 VDRRC; three of them have been generated in S Eaton, CWT or JM laboratories (Stock column). *UAS-RNAi* lines that produced a sterile phenotype were re-used with the *promE-gal4* driver, except 6660R-2 used to knockdown *elo^{CG6660}*.

Males CHC	<i>P</i>	<i>6432i-C</i>	<i>6432i</i>
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 ± 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	<i>P</i>	<i>6432i-C</i>	<i>6432i</i>
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 ± 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-H	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.