Title

Insulin/insulin-like growth factor signaling pathway promotes higher fat storage in *Drosophila* females

Authors

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Summary

In *Drosophila*, adult females store more fat than males. While the mechanisms that restrict body fat in males are becoming clearer, less is known about how females achieve higher fat storage. Here, we perform a detailed investigation of the mechanisms that promote higher fat storage in females. We show greater intake of dietary sugar supports higher fat storage due to female-biased remodeling of the fat body lipidome. Dietary sugar stimulates a female-specific increase in *Drosophila* insulin-like peptide 3 (Dilp3), which acts together with greater peripheral insulin sensitivity to augment insulin/insulin-like growth factor signaling pathway (IIS) activity in adult females. Given that adult-specific IIS inhibition reduced the sex difference in fat storage due to a female-biased decrease in body fat, our data reveal IIS as a key determinant of female fat storage.

Keywords

Sex difference, body fat, triglyceride, insulin/insulin-like growth factor signaling pathway, sugar, insulin sensitivity

Introduction

In *Drosophila*, as in other animals, triglyceride is the main stored form of fat[1]. The primary depot for stored fat in insects is called the fat body[1]; however, other cell types and organs in the body also have the capacity to synthesize and store triglyceride (*e.g.*, gut, oenocytes, neuron, glia, muscle, testis, ovary)[2-9]. While many factors influence the total amount of stored fat, one important determinant of body fat in flies is biological sex[5,9,10]. For example, *Drosophila* virgin females store more fat than males[5], a difference that is amplified post-mating[9,11]. Virgin female flies also break down fat more slowly than males after nutrient withdrawal[5]. These sex differences in body fat regulation are physiologically relevant for reproduction. In females, greater fat stores are needed to support the energetic demands of reproduction[9,12] and to survive long periods without food[5]. In males, restricting excess triglyceride accumulation is important to maintain fertility[8].

Over the past two decades, studies in *Drosophila* have played a key role in identifying many genes and pathways that contribute to whole-body fat storage. For example, loss-of-function mutations or RNAi-mediated knockdown of genes predicted to promote triglyceride synthesis lead to reduced fat storage in both individual cells and in the whole body[12-16]. In contrast, genetic inhibition of genes predicted to facilitate triglyceride breakdown cause a significant increase in fat storage at the cellular and organismal levels[17-19]. Gain- and loss-of-function experiments with genes predicted to regulate lipid droplet dynamics, a specialized organelle dedicated to triglyceride storage, similarly influence cellular and organismal fat stores[16,19-24]. Beyond these enzymes directly involved in triglyceride metabolism and lipid droplet biology, large-scale screening efforts have revealed many additional genes that influence *Drosophila* body fat[4,18,25-39].

In addition to the action of individual genes, a large body of literature has identified many hormones and peptides that influence whole-body fat storage[9,40-57]. In many cases, these hormones modulate whole-body fat storage via effects on cells that produce

59 adipokinetic hormone (Akh)[43-46,58] and *Drosophila* insulin-like peptides (Dilps)[30,43,45,59-62].
60 Akh and Dilps have opposing effects on whole-body fat storage[63]. Under conditions where
61 nutrients are plentiful, Dilps are released from the median neurosecretory cells in the
62 *Drosophila* brain into the hemolymph. Binding of the Dilps to the insulin receptor (InR) on
63 the fat body stimulates the insulin/insulin-like growth factor signaling pathway (IIS) to
64 promote fat storage[53]. When nutrients are scarce, Akh is released into the hemolymph from
65 the Akh-producing cells in the *corpora cardiaca*[46,64-67]. Akh binds to the Akh receptor (Akhr)
66 located in the fat body where it promotes fat breakdown[55-57]. While studies on specific genes
67 and hormones have advanced knowledge of the mechanisms underlying the regulation of
68 fat storage, most studies used single- or mixed-sex animal groups. As a result, we know
69 less about the mechanisms underlying sex differences in fat storage.

70 Recent studies have begun to close this knowledge gap. Both large-scale RNAseq
71 data and targeted gene expression studies reveal profound sex differences in expression of
72 genes known to influence cellular and whole-body fat storage[5,68,69]. For example, male flies
73 express higher mRNA levels of the *Drosophila* homolog of adipose triglyceride lipase called
74 *brummer* (*bmm*), an enzyme that limits triglyceride accumulation across most cell types[17].
75 This male-biased *bmm* expression is physiologically significant, as whole-body loss of *bmm*
76 abolishes the sex difference in fat storage via a male-biased increase in body fat[5]. A similar
77 male bias has also been reported in the production and secretion of Akh[10]. Specifically,
78 males produce and secrete more Akh than females under basal conditions, which prevents
79 fat accumulation to drive lower body fat in males[10]. A sex bias in two central lipolytic
80 pathways therefore plays a key role in establishing the sex difference in body fat by
81 restricting triglyceride accumulation in males. Yet, the mechanisms that allow increased fat
82 storage in females remain less clear, as females are largely unaffected by loss of *bmm* and
83 Akh[10].

84 In mated females, higher levels of steroid hormone ecdysone enhance body fat by
85 stimulating food intake[9]. Adult mated females also show higher mRNA levels of Dilp3[70],
86 where Dilp3 has been implicated in promoting fat storage[43,62]. Whether these mechanisms
87 also influence the sex difference in fat storage remains unclear. We therefore performed a
88 detailed analysis of adult virgin males and females to explore how females achieve a higher
89 level of fat storage. We found that virgin female flies normally consume more food than
90 males, and show that female fat storage is sensitive to changes in food quantity. In particular,
91 consumption of dietary sugar and not simply a greater number of calories explained this
92 higher fat storage in females. Unbiased lipidomic and metabolomic analysis of abdominal
93 carcasses from adult flies revealed dietary sugar caused a profoundly female-biased
94 metabolic remodeling of energy-storing cells including the fat body. Given that ecdysone did
95 not promote increased body fat in virgin females, we characterized sex differences in IIS,
96 the other key pathway that promotes fat storage. We identified a significant female bias in
97 Dilp3 mRNA levels and fat body insulin sensitivity, leading to increased IIS activity in the
98 female fat body. This sex bias in IIS was significant in promoting female fat storage, as
99 ablating the Dilp-producing cells in adults caused a female-specific reduction in body fat.
100 Together, these data identify IIS as a key pathway that promotes higher fat storage in adult
101 females.
102
103 **Results**
104 **Dietary sugar promotes increased fat storage in adult females**
105 To determine whether unmated females have higher food intake than males we used the
106 capillary feeder (CAFE) assay[71] to assess food consumption in 5-day-old *Drosophila* virgin
107 males and females. Five-day-old adult *Canton-S* (*CS*) females consumed significantly more
108 food per body weight over 24 hr than genotype-matched males (Figure 1A), a trend we
109 reproduced in 5-day-old w^{1118} adults (p=0.0812; Figure S1A). Considering changes in food

110 intake affect body fat[26,47,72], this suggests higher food consumption may explain this
111 increased fat storage in females. Indeed, reducing nutrient content in the diet significantly
112 reduced body fat in *CS* females (Figure 1B). Because body fat in *CS* males was unaffected
113 by an identical food dilution (Figure 1B), the sex difference in body fat was reduced. We next
114 tested the contribution of specific diet components to higher fat storage in females. Given
115 that dietary sugar affects body fat in a mixed-sex population of *Drosophila*[73-77], we
116 transferred newly-eclosed *CS* and w^{1118} flies to a diet lacking added sugar (0S) for 5 days[78].
117 In both w^{1118} and *CS* strains, adult females transferred to the 0S diet had significantly less
118 body fat compared with genotype-matched flies kept on a widely-used lab diet with sugar
119 (1S; Figure 1C and S1B)[79]. Because the magnitude of the reduction in body fat was smaller
120 in w^{1118} males (Figure 1C; sex:genotype interaction $p<0.0001$), with the same trend in *CS*
121 males (Figure S1B), the sex difference in fat storage was reduced.

122 The female-biased reduction in fat storage on 0S cannot be attributed to altered food
123 intake on the low-sugar diet, as food consumption was not different between flies kept on
124 the 0S diet compared with flies transferred to the 1S diet (Figure S1C). A decrease in
125 calories similarly cannot explain the effect of reduced sugar on body fat, as female flies
126 transferred to a reduced-calorie diet with sugar still had higher body fat than females kept
127 on the 0S diet (Figure 1D). Because neither sucrose nor D-glucose on their own were
128 sufficient to restore fat storage to the level observed in females maintained on the 1S diet
129 (Figure 1E), our data suggests it is the total amount of sugar rather than a specific sugar
130 that promotes increased fat storage in female flies. Indeed, providing females with more
131 sugar is sufficient to augment body fat even further (Figure 1F). Supporting a model in which
132 the nutritive value of sugar rather than sweet taste promotes increased female fat storage,
133 body fat was not higher in either male or female flies kept on the 0S diet containing a non-
134 nutritive but sweet-tasting sugar[80] (Figure S1D). Thus, our data suggest that higher food

135 intake, specifically increased consumption of dietary sugar, promotes higher fat storage in
136 females.
137
138 **Female-biased effect of dietary sugar on metabolism**
139 To assess how sex and dietary sugar affect the lipidome and metabolome of fat-storing
140 organs in *Drosophila*, we performed untargeted mass spectrometry (MS)-based lipidomic
141 profiling on abdominal carcasses isolated from adult male and female flies transferred to
142 either a 1S or 0S diet as adults. We detected 464 lipid species in total (Supplemental Table
143 S1). We first compared lipid abundance between males and females. We identified sex
144 differences in the abundance of 163 lipids in flies maintained on the 1S diet (p<0.01): 117
145 lipid species were significantly higher in females and 46 lipid species were significantly
146 higher in males (Figure 2A). Triglyceride was the lipid subclass showing the strongest female
147 bias, with 48/57 (86%) species significantly higher in females compared with males (Figure
148 2B). This supports increased stored fat in females. When we examined the chain length of
149 fatty acids in the triglyceride pool, we found that females had a greater percentage of
150 monounsaturated fatty acids such as myristoleic acid (C14:1), palmitoleic acid (C16:1), and
151 oleic acid (C18:1) in the triglyceride pool (Figure S2A). Females also showed a greater
152 percentage of most odd-chain fatty acids in the triglyceride pool (Figure S2A), with a similar
153 female bias in C28:2 (7/7). In males, there was a strong bias in levels of C24:1 (4/4) in the
154 triglyceride pool (Figure S2A). Beyond triglyceride, we also noted a significant female bias
155 in lipids such as acyl-carnitine (acyl-Car) (4/5), ceramide (Cer) (4/7), diacylglycerol (DG)
156 (6/9), phosphatidylcholine (PC) (16/25), ether-linked phosphatidylcholine (PC-O) (4/6),
157 phosphatidylethanolamine (PE) (17/20), phosphatidylglycerol (PG) (3/3),
158 phosphatidylinositol (PI) (3/3), sterol-ester (SE) (4/4) and sterol (ST) (4/4) (Figure 2B). Lipids
159 with a significant male bias included lysophosphatidylcholine (LPC) (2/3) and ether-linked
160 triacylglycerol (TG-O) (12/13) (Figure 2B). As with lipids, there was a significant female bias

161 in many metabolites ($p<0.05$) (Figure 2C), including asparagine, glutamine, glutamic acid,
162 glucose-6-phosphate, ADP, propionylcarnitine, pantothenic acid, which together suggest a
163 female bias in glycolysis and the tricarboxylic acid (TCA) cycle. Thus, our data show that
164 biological sex is a key regulator of the lipidome and metabolome.

165 To determine how dietary sugar affects the lipidome and metabolome in each sex,
166 we compared these parameters in abdominal carcasses of flies transferred to the 1S or 0S
167 diets as adults. In females, we observed significant differences in levels of 218 lipids
168 between flies transferred to 1S compared with flies out on 0S ($p<0.01$). Specifically, dietary
169 sugar was associated with an increased abundance of 154 lipids and reduced abundance
170 of 64 lipids (Figure 2D). Triglyceride showed the largest sugar-dependent change in
171 abundance, where females maintained on 0S had significantly lower triglyceride abundance
172 than females kept on 1S (61/81) (Figure S2B-D), consistent with our triglyceride assay data.
173 Females maintained without sugar had a lower percentage of many fatty acids, such as
174 C16:1 and C18:1, in the triglyceride pool compared with females kept on the 1S diet (Figure
175 S2E). A similar reduction in the abundance of C28:2 in the triglyceride pool was also
176 observed in females maintained on 0S (Figure S2E). Broader effects of reduced dietary
177 sugar were also observed across many lipid subclasses in females: Acyl-Car (3/4), Cer (5/9),
178 DG (11/17), PC (29/33), PC-O (4/7), PE (25/27), PG (5/5) (Figure S2B). Lipids that were
179 significantly higher in abundance in females maintained on the 0S diet included PI species
180 (2/6) and TG-O (4/21) (Figure S2B). Because body size is fixed before the adult stage of
181 development[81], and the females are only fed 1S or 0S after eclosion, these diet-induced
182 alterations in lipid abundance cannot be explained by a sugar-dependent change in body
183 size. In males, only 27 lipid species were differentially regulated by dietary sugar: 17 lipids
184 were higher in abundance in flies maintained on a 1S diet whereas 10 lipids were lower in
185 abundance (Figure 2E and S2F). Lipids that were lower in abundance in males on the 0S
186 diet included PC (7/7), PC-O (2/3), triglyceride (4/6) (Figure S2G).

187 This female bias in sugar-induced changes in the fat body was reproduced in our
188 metabolomic data (Supplemental Table S1, Figure 2F): 38 metabolites showed a difference
189 in abundance in females maintained on the 0S diet whereas only 13 metabolites were
190 altered in males kept on 0S (p<0.05 threshold for both sexes) (Figure 2F). Metabolites that
191 were altered in females on the 0S diet indicate reduced glycolysis and TCA cycle
192 (asparagine, glutamine, glutamic acid, glucose-6-phosphate, ADP, nicotinamide,
193 propionylcarnitine, pantothenic acid) and lower oxidative stress (oxidized glutathione, tans-
194 4-hydroxy-L-proline, methioninesulfoxide, L-5-oxoproline (also known as pyroglutamic acid),
195 kynurenine) (Figure 2F). In males, metabolites that were altered suggest reduced glycolysis
196 on the 0S diet (nicotinamide, NAD, AMP) (Figure 2G). Dietary sugar therefore caused a
197 strongly female-biased remodeling of the lipidome and metabolome in abdominal carcasses.
198 While the reason for this profound female bias in changes to the lipidome and metabolome
199 in response to dietary sugar is unclear, males showed higher levels of sugar excretion on
200 the 1S diet (Figure 2H).
201
202 **Dietary sugar promotes sex differences in IIS regulation**
203 Considering dietary sugar stimulates the production and secretion of Dilps from the insulin-
204 producing cells (IPC) in the brain[82-85], we tested if Dilp production, Dilp secretion, and IIS
205 activity differed between males and females maintained on 1S. We first measured mRNA
206 levels of IPC-derived *dilp2*, *dilp3* and *dilp5* in adult heads using quantitative real-time
207 polymerase chain reaction (qPCR). Female heads had higher mRNA levels of *dilp3* than
208 male heads (Figure 3A); no significant differences in mRNA levels of *dilp2* and *dilp5* were
209 found between the sexes (Figure 3B and 3C). To test whether dietary sugar caused the sex
210 difference in *dilp3* mRNA levels, we compared *dilp3* mRNA levels in males and females
211 transferred to either 1S or 0S diets as adults. Female flies transferred to 0S had significantly
212 lower *dilp3* mRNA levels than females kept on 1S (Figure 3D). Because there was no sugar-

213 dependent change in male *dilp3* levels, the sex difference in *dilp3* levels was eliminated
214 (Figure 3D; sex:diet interaction p=0.0128). Our data therefore suggest dietary sugar plays a
215 role in the female bias in *dilp3* mRNA levels. We next monitored Dilp secretion from the IPCs
216 in the brain by expressing a calcium-responsive chimeric transcription factor LexA-VP16-
217 NFAT (*UAS-LexA-VP16-NFAT* [called *UAS-CaLexA*]) specifically in these cells using *dilp2-*
218 *GAL4* (full genotype *dilp2-GAL4>UAS-LexA-VP16-NFAT*). This chimeric transcription factor
219 drives expression of GFP in response to changes in cellular calcium levels, providing an
220 indirect readout for neuronal activity and consequently Dilp release[86]. On the 1S diet, there
221 was no significant difference in GFP expression between males and females in the IPC
222 (Figure 3E). This suggests Dilp release was equivalent between the sexes, confirming
223 results from a very recent study showing IPC activity does not differ between the sexes[87].
224 Given the female bias in *dilp3* mRNA levels, and the known role of *dilp3* in promoting
225 IIS activity, we next compared IIS activity in males and females. To assess IIS activity, we
226 measured mRNA from known transcriptional targets of Forkhead box, sub-group O (Foxo;
227 FBgn0038197) in male and female abdominal carcasses. When IIS activity is high, Foxo is
228 phosphorylated and repressed, leading to lower mRNA levels of its known target genes *InR*,
229 *bmm*, and *eukaryotic initiation factor 4E-binding protein* (*4E-BP*)[88-91]. In adult males and
230 females maintained on 1S, mRNA levels of Foxo target genes were significantly lower in
231 females than in males (Figure 3F). This suggests IIS activity is normally higher in females
232 than in males. Given that *Drosophila* female larvae are more insulin sensitive than male
233 larvae[78], a sex difference that also exists across many tissues in mammals[92,93], we also
234 compared insulin sensitivity between adult virgin males and females kept on 1S. Considering
235 female abdominal carcasses showed significant insulin-induced repression of Foxo target
236 genes (Figure 3G), a response we did not observe in males (Figure 3H), our data suggest
237 female abdominal carcasses are more insulin sensitive than males. Taken together, our data
238 show that dietary sugar promotes higher expression of *dilp3* in females. Increased *dilp3*,

239 combined with higher insulin sensitivity in females, likely contribute to elevated IIS activity in
240 females compared with males.
241
242 **Females require IIS to maintain fat storage**
243 Insulin is a potent lipogenic hormone in multiple animals including *Drosophila*[53,94-98].
244 Increased IIS activity in female abdominal carcasses may therefore explain their higher fat
245 storage. Because reduced IIS activity during development significantly impairs larval growth
246 and survival to adulthood[54,99-104], and increased IIS activity during development augments
247 body size[103-106], we needed a system to modulate IIS activity specifically in adults.
248 Unfortunately, existing systems for inducible gene expression have undesirable limitations
249 for studying sex differences in fat metabolism. The Gene Switch system[107,108] induces
250 transgene expression by feeding flies mifepristone, which has sex-specific effects on adult
251 physiology[109-111]. The AGES (Auxin-inducible Gene Expression System) uses auxin feeding
252 to induce transgene expression[112], which we and others recently showed affects feeding
253 and triglyceride metabolism in a sex-biased manner[113].
254 Another inducible system for gene expression, the temporal and regional gene
255 expression targeting (TARGET) system[114], normally requires long-term temperature shifts
256 at 29°C to inactivate temperature-sensitive repressor GAL80. Given that inactive GAL80
257 cannot repress GAL4, UAS transgene-mediated expression is initiated as long as the
258 animals are kept at 29°C. Unfortunately, 29°C is a temperature known to disrupt lipid
259 metabolism[115,116], making a long-term heat shock to induce transgene expression or RNAi-
260 mediated knockdown undesirable. To overcome this limitation, we reasoned that we could
261 deliver a brief 3 hr heat shock to newly-eclosed male and female flies to transiently express
262 proapoptotic gene *reaper* (*rpr*) specifically in the IPC (genotype: *dilp2-GAL4>UAS-rpr,tub-*
263 *GAL80^{ts}*). We expected this approach would ablate the IPC specifically in adults. Supporting
264 successful IPC ablation, mRNA levels of *dilp2*, *dilp3* and *dilp5* were significantly lower in

265 adult heads from female and male flies with a brief heat shock compared with genotype-
266 matched controls without heat shock (Figure 4A and 4B). Additional evidence supporting
267 effective IPC ablation was the extremely long lifespan of the IPC-ablated female and male
268 flies (Figure 4C and 4D)[117-122] and the reduced fertility in female flies (Figure 4E)[123]. IPC
269 ablation in male flies had no effect on fertility (Figure 4F).

270 Using this system to ablate the IPC, we tested whether increased IIS was required
271 for females to store more body fat than males. We found that triglyceride levels were
272 significantly lower in 5-day-old females with ablated IPCs (genotype *dilp2-GAL4>UAS-*
273 *rpr,tub-GAL80^{ts}*) than in control females (genotypes *dilp2-GAL4>+,tub-GAL80^{ts}* and *+>UAS-*
274 *rpr*) (Figure 4G). In contrast, IPC ablation in males had no significant effect on body fat
275 (Figure 4G). We also found that females (genotype *dilp2-GAL4>UAS-rpr,tub-GAL80^{ts}*)
276 subjected to a brief heat shock had lower body fat compared with genotype-matched
277 females that were not heat-shocked (Figure 4H). While heat-shocked *dilp2-GAL4>UAS-*
278 *rpr,tub-GAL80^{ts}* males showed a modest decrease in body fat (Figure 4H), the magnitude of
279 the decrease was greater in females (sex:genotype interaction p=0.0258). The reduction in
280 female body fat was not explained by the brief heat shocks, as control females (genotype
281 *dilp2-GAL4>w^{1118},tub-GAL80^{ts}*) subjected to an identical heat shock showed no significant
282 effect on body fat (Figure 4I). The decrease in body fat was also not due to altered feeding
283 in animals with IPC ablation, as no significant differences in food consumption were present
284 in either females or males with IPC ablation compared with control flies (Figure 4J and 4K).
285 Supporting a specific role for IIS in maintaining higher fat storage in females, supplementing
286 the diet of virgin females with an ecdysteroid called 20-hydroxyecdysone (20HE) did not
287 promote fat storage in females (Figure S3A). Our data therefore reveal sex differences in
288 IIS regulation in adult flies, and demonstrate the importance of this regulation to the male-
289 female difference in fat storage.
290

291 **Discussion**

292 Our findings provide significant insight into the mechanisms by which adult females store
293 more fat than males. This information is critical to our understanding of how sex differences
294 in body fat are established and maintained, as past studies identified mechanisms that
295 operate in males with little effect in females[5,10]. Based on our data, we propose a model in
296 which higher consumption of dietary sugar in females leads to elevated *dilp3* mRNA levels,
297 which act together with greater insulin sensitivity to promote higher IIS activity and body fat
298 in females. Supporting this model, reduced sugar consumption or IPC ablation lessened the
299 sex difference in fat storage by lowering body fat in females with little effect in males. This
300 reveals previously unrecognized sex-specific IIS regulation in adult flies, and identifies IIS
301 as a key pathway that supports increased fat storage in adult females.

302 One key advance of our study was to extend knowledge of how food intake affects
303 physiology in unmated females. Past studies used mated females to show that greater food
304 consumption supports the quality and quantity of eggs produced[9,124-131]. While greater total
305 nutrient intake could explain these effects, specific macronutrients such as dietary protein
306 play individual roles in supporting egg production, egg-laying, and postcopulatory behaviors
307 in mated females[132-134]. Indeed, dietary protein is limiting for *Drosophila* egg production[135],
308 where its biological importance is shown by the fact that mated females will adjust their food
309 preferences to ensure adequate protein intake[133,136,137]. Dietary protein does not hold the
310 same physiological value for unmated females, however, as virgin females do not adjust
311 their preferences after protein restriction[137]. Instead, our data suggest that dietary sugar is
312 a key macronutrient for virgin female physiology due to its effects on IIS activity and fat
313 accumulation. Because IIS activity supports normal germline stem cell proliferation and
314 maintenance in the ovary[54,123,135,138-141], and high levels of body fat are required for
315 successful egg production[12], dietary sugar consumption likely helps to establish a supportive
316 metabolic state prior to reproduction. Once mated, this role for dietary sugar becomes less

317 important, as reflected by the limited consumption of a sugar-rich diet in mated females
318 compared with males[142]. Thus, our data suggests that higher food intake in both virgin and
319 mated females provides macronutrients to fuel the specific needs that accompany particular
320 life history stages (*e.g*. prior to mating or during egg production).

321 Another advance of our study was to reveal a profound sex difference in how dietary
322 sugar affects metabolism in the fat body. In females, sugar caused profound remodeling of
323 the lipidome and many changes in the metabolome. This sex difference was most apparent
324 when examining triglyceride, the lipid sub-class that shows the most obvious sugar-
325 dependent changes in female lipid abundance. However, females showed a sugar-
326 dependent increase across many lipid sub-classes (*e.g*., DG, PG, PE, Acyl-Car). Given that
327 females have lower lipase activity than males[5], the presence of high diglyceride and
328 triglyceride levels suggests increased lipid synthesis in females. Diglyceride is also a
329 precursor for phospholipids such as PE and PC synthesis[1,143]. High levels of PE and PC in
330 females maintained on 1S further support higher lipid synthesis in females, a possibility that
331 will be important to test in future studies.

332 Other than this broad lipid accumulation in females kept on 1S, other interesting
333 findings were that dietary sugar enhanced levels of a fatty acid precursor used for synthesis
334 of female-specific pheromones (28:2) and odd-chain fatty acids in the triglyceride pool. Fatty
335 acid 28:2 is the direct precursor for the synthesis of a cuticular hydrocarbon called 7,11-
336 heptacosadiene, the major female pheromone that mediates female sexual
337 attractiveness[144-146]. Thus, in addition to supporting IIS activity and fat accumulation in
338 females, dietary sugar consumption may also ensure females emit an optimal pheromone
339 profile. Indeed, sexual attractiveness is altered in females with abnormal levels of cuticular
340 hydrocarbons[147]. Given that the gut microbiota help produce odd-chain fatty acids[148] and
341 the microbe load is higher in adult female intestines than in males[149], our data suggest that
342 the intestinal microbiome may provide precursors used for fat storage in females exposed

343 to dietary sugar. Thus, dietary sugar provides an array of physiological benefits for females
344 that likely optimize reproduction.

345 In males, there were few changes in lipids or metabolites due to changes in dietary
346 sugar. One notable finding from our comparison of differentially-regulated lipids between
347 males and females was that only one lipid subclass, TG-O, was more abundant in males
348 than in females. Ether-linked lipids such as TG-O are synthesized by a multi-step process
349 mediated by enzymes located both in the peroxisome and endoplasmic reticulum[150-152].
350 Higher levels of TG-O in males may indicate sugar-induced lipotoxicity in males, as a recent
351 study found high levels of TG-O were induced in male flies chronically fed a high-sugar
352 diet[153]. In that study, elevated TG-O levels were associated with metabolic dysfunction, as
353 genetically reducing production of ether-linked lipids in male flies reversed defects in glucose
354 levels, cardiac function, and insulin signaling caused by a high-sugar diet[153,154]. Future
355 studies will therefore need to examine ether-linked lipid synthesis in both sexes to determine
356 whether male-female differences exist in this process and its relationship to metabolic
357 dysfunction. Follow-up studies will also need to determine the ultimate reason that males
358 largely fail to adjust the lipidome in response to dietary sugar. Potential explanations
359 suggested by our data include insulin resistance, increased sugar excretion, and high lipase
360 and lipolytic pathway activity[5,10]. Published data suggest that greater carbohydrate
361 breakdown within the male gut to support spermatogenesis might also explain the female-
362 biased lipidomic response to dietary sugar[155].

363 Other key findings of our study include showing that dietary sugar promotes a female
364 bias in *dilp3* levels, and revealing a female bias in IIS activity within the adult fat body. This
365 extends knowledge from past studies on sex differences in IIS activity in third-instar larvae[103]
366 by showing this female bias in IIS activity is present in adults and is physiologically
367 significant. Given that elevated *dilp3* levels are known to promote body fat[43,62,156] and
368 prolong starvation resistance[61,157,158], the female bias in *dilp3* is significant for higher fat

369 accumulation in females. However, the mechanisms underlying the sex bias in *dilp3* levels
370 remain unclear. Because the IPCs do not directly sense sugar[85], dietary sugar likely does
371 not directly act on the IPCs to influence the sex difference in *dilp3* levels. Instead, future
372 studies will need to determine whether *dilp3*-regulatory factors identified in single- and
373 mixed-sex studies such as gut-derived neuropeptide F[43], octopamine[157], tachykinin[159],
374 leucokinin[61,160], adiponectin receptor[161], Allatostatin A[45], bursicon[62], drosulfakinin[158], and
375 potentially Akh and Limostatin[156,162,163], contribute to the sex difference in *dilp3* mRNA
376 levels. Indeed, sex differences in expression of multiple *dilp3*-regulatory factors have been
377 reported[10,164,165], where these factors have sex-specific effects on physiology[10,42-
378 46,61,62,156,157,159] and influence mating and/or reproductive behaviors[166-174].

379 Overall, our findings identify IIS as a major determinant of female fat storage, with
380 only minor effects on male body fat. This contrasts with males, where a set point of stored
381 fat is maintained by *bmm* and Akh, pathways with little effect on female body fat[5,10]. The
382 primary mechanisms that establish fat storage are therefore not identical between the sexes:
383 females sustain body fat primarily via the anabolic effects of insulin whereas body fat in
384 males is maintained by the lipolytic effects of *bmm* and Akh. Future studies will need to
385 determine whether the sex-biased effects of changing IIS and Akh on body fat are
386 reproduced in other aspects of physiology regulated by these pathways (*e.g*. locomotion,
387 lifespan, aggression, feeding, sleep)[64,122,123,126,175-179]. Future studies will also need to
388 determine whether the extensive crosstalk between IIS and Akh identified in single- and
389 mixed-sex studies in adults and larvae is shared between males and females[45,57,99,163,180].
390 Because IIS and Akh mediate responses to a variety of extrinsic and intrinsic cues, gaining
391 a better understanding of the interplay between these two major hormone pathways in males
392 and females will provide critical insights into how diverse cues are integrated to maintain
393 whole-body triglyceride homeostasis in each sex. Given that sex differences in sucrose-
394 induced changes to mammalian adipose tissue lipolysis, hepatic lipogenesis, and adipose

395 tissue gene expression broadly reproduce the effects we report in flies[181], the sex-biased
396 effects of IIS and Akh on body fat we observed have implications for our understanding of
397 sex differences in parallel hormone systems in mammals (*e.g*. insulin, glucagon) and the
398 sex-biased risk of diseases associated with these hormones[182-186].
399
400 **Resource availability**
401 **Lead contact**
402 Further information and requests for resources and reagents should be directed to and will
403 be fulfilled by the lead contact, Elizabeth J. Rideout (elizabeth.rideout@ubc.ca).
404 **Materials availability**
405 This study did not generate new unique reagents.
406 **Data and code availability**
407 • Fly lipidomic and metabolomic data are available in Supplemental Table S1.
408 • This paper does not report original code.
409 • Any additional information required to reanalyze the data reported in this paper is available
410 from the lead contact upon request.
411
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431 **Author contributions**

432 P. B. conceived studies, collected samples, conducted experiments, interpreted
433 experiments, wrote and edited the manuscript.

434 H. Y. performed lipidomic and metabolomic experiments.

435 L. W. W. conducted ecdysone experiment.

436 C. J. M. conducted brain dissection and IPC activity.

437 T. H. edited the manuscript and acquired funding for lipidomic and metabolomic analyses.

438 E. J. R. conceived studies, interpreted experiments, wrote and edited the manuscript,
439 acquired funding, and is the guarantor of this work.

440

441 **Declaration of interests**

442 The authors declare no competing interests.

443

444 **Supplemental information**

445 Table S1. Excel file containing raw data with calculations and primer list

446 Table S2. Excel file containing statistics for all data

447 **Figure legends**

448 **Figure 1. Dietary sugar promotes increased fat storage in adult females.**

449 (A) Food consumption per unit of body weight was significantly higher in 5-day-old adult
450 *Canton-S (CS)* females than males (p=0.0072; Student's t-test); n=8 biological replicates.

451 (B) Whole-body triglyceride levels were significantly lower in 5-day-old *CS* females
452 maintained on a diet with reduced nutrients (20% of normal nutrient content) compared with
453 females kept on our normal diet (100% diet; p<0.0001). No change in whole-body
454 triglyceride was observed in male flies (p>0.9999; sex:diet interaction p<0.0001). Two-way
455 ANOVA followed by Bonferroni post-hoc test; n=16 biological replicates.

456 (C) Whole-body triglyceride levels were significantly higher in 5-day-old adult w^{1118} females
457 and males maintained on the 1S diet compared with sex-matched flies kept on the 0S diet
458 (Females: p<0.0001; Males: p=0.0195). The magnitude of the decrease in body fat was
459 greater in females than in males (sex:diet interaction p<0.0001).Two-way ANOVA followed
460 by Bonferroni post-hoc test; n=16 biological replicates.

461 (D) Whole-body triglyceride levels were significantly higher in 5-day-old adult w^{1118} females
462 maintained on sugar-containing diet with reduced calories (1S-LC) compared with females
463 kept on the 0S diet (same calorie content as 1S-LC) (p=0.0032); no significant change in
464 body fat was observed in males (p=0.3715; sex:diet interaction p=0.0019). Two-way ANOVA
465 followed by Bonferroni post-hoc test; n=8 biological replicates.

466 (E) Whole-body triglyceride levels were not significantly different between 5-day-old adult
467 w^{1118} females maintained on the 0S diet and sex-matched flies kept on the 0S diet with either
468 added sucrose or D-glucose diet (females: p=0.9998 (sucrose), p=0.7532 (D-glucose). One-
469 way ANOVA followed by Tukey HSD test; n=8 biological replicates.

470 (F) Whole-body triglyceride levels were significantly higher in 5-day-old adult w^{1118} females
471 maintained on our lab diet with 30% added sugar (HSD) compared with females kept on the
472 1S diet (p=0.0015; Student's t-test). n=8 biological replicates.

473 All data plotted as mean ± SEM. ns indicates not significant with p>0.05; * p<0.05, ** p<0.01,
474 *** p<0.001, **** p<0.0001.

475 See also figure S1.

476

477 **Figure 2. Female-biased effect of dietary sugar on metabolism.**
478 (A) Volcano plot of differentially-regulated lipid species between 5-day-old adult female and
479 male w^{1118} fat bodies in 1S diet (colored dots represent p<0.01; Student's t-test).
480 (B) Proportion of species in each lipid subclass with a sex bias in abundance on the 1S diet
481 (p<0.01; Student's t-test). Numbers on histograms indicate the number of lipid species with
482 differential abundance.
483 (C) Sex bias of all metabolites with differential abundance between female and male fat
484 bodies in 1S diet (p<0.05; Student's t-test).
485 (D) Volcano plot of lipid species that are differentially-regulated between females maintained
486 on either the 1S or the 0S diet (colored dots represent p<0.01; Student's t-test).
487 (E) Volcano plot of lipid species that are differentially-regulated between males maintained
488 on either the 1S or the 0S diet (colored dots represent p<0.01; Student's t-test).
489 (F) Volcano plot of metabolites that are differentially-regulated between females maintained
490 on either the 1S or the 0S diet (colored dots represent p<0.05; Student's t-test).
491 (G) Volcano plot of metabolites that are differentially-regulated between males maintained
492 on either the 1S or the 0S diet (colored dots represent p<0.05; Student's t-test).

493 (H) Glucose levels in the excreta of 6-day-old adult w^{1118} males were higher than females
494 (p=0.0461, Student's t-test); n=4-6 biological replicates. Data plotted as mean ± SEM. *
495 p<0.05
496 TG: triglyceride; TG-O: ether-linked TG; DG: diglyceride; PI: phosphatidylinositol; PC:
497 phosphatidylcholine; PC-O: ether-linked PC; ST: sterol; LPC: lyso-PC; PG:
498 phosphatidylglycerol; PS: phosphatidylserine; PE: phosphatidylethanolamine; PE-O or PE-
499 P: ether-linked PE; NAE: N-acetylethanolamine; Cer: Ceramide; Acyl-Car: acyl-carnitine;
500 SE: sterol ester; Hex-Cer: hexosyl-ceramide.
501 See also figure S2.
502
503 **Figure 3. Dietary sugar promotes sex differences in IIS regulation.**
504 (A) mRNA levels of IPC-derived *dilp3* in heads were significantly higher in 5-day-old w^{1118}
505 females compared with males (p<0.0001; Student's t-test); n=7 biological replicates.

506 (B, C) mRNA levels of IPC-derived *dilp2* (B) and *dilp5* (C) in heads were not significantly
507 different between 5-day-old w^{1118} males and females (p=0.1282, Mann-Whitney test (*dilp2*);
508 p=0.5753, Student's t-test (*dilp5*)); n=7 biological replicates.

509 (D) mRNA levels of IPC-derived *dilp3* in heads were significantly lower in females
510 maintained on the 0S diet compared to females kept on the 1S diet (p<0.0001). There was
511 no sugar-dependent change in *dilp3* mRNA in males (p=0.4809; sex:diet interaction
512 p=0.0128). Two-way ANOVA followed by Bonferroni post-hoc test; n=7 biological replicates.
513 Note: *dilp3* mRNA levels in 1S diet were replotted from Figure 3A as these data were
514 collected in the same experiment.

515 (E) Quantification of GFP intensity produced by expression of *UAS-CaLexA* in the IPCs
516 (*dilp2-GAL4>UAS-CaLexA,UAS-mRFP*), normalized to RFP levels. Normalized GFP

517 intensity was not significantly different between females and males in 1S diet (p=0.4782;
518 Mann-Whitney test); n=15-23 animals.

519 (F) mRNA levels of Foxo target genes (*InR, bmm,* and *4E-BP*) in fat bodies were significantly
520 higher in 5-day-old w^{1118} males compared to females in 1S diet (p=0.0240; Mann-Whitney
521 test); n=7 biological replicates.

522 (G) mRNA levels of Foxo target genes (*InR, bmm*, and *4E-BP*) in 5-day-old w^{1118} female fat
523 bodies were significantly reduced after stimulation with 1 μM insulin compared with fat
524 bodies lacking insulin stimulation (p<0.0001; Student's *t*-test); n=6 biological replicates.

525 (H) mRNA levels of Foxo target genes *(InR, bmm*, and *4E-BP*) in 5-day-old w^{1118} male fat
526 bodies were not significantly different between unstimulated and insulin-stimulated
527 conditions (p=0.5841; Mann-Whitney test); n=6 biological replicates.

528 All data plotted as mean ± SEM. ns indicates not significant with p>0.05; * p<0.05, ** p<0.01,
529 *** p<0.001, **** p<0.0001.

530

531 **Figure 4. Females require IIS to maintain fat storage.**

532 (A, B) mRNA levels of IPC-derived *dilp2, dilp3, dilp5* in heads were significantly lower in both
533 female (A; p<0.0001, Student's *t*-test) and male (B; p=0.0016, Mann-Whitney test) *dilp2-*
534 *GAL4>UAS-rpr,tub-GAL80*^{ts} flies with heat shock at 29°C compared to sex-matched flies
535 reared in room temperature; n=4-6 biological replicates.

536 (C) Lifespan was significantly longer in *dilp2-GAL4>UAS-rpr,tub-GAL80*^{ts} females compared
537 with *dilp2-GAL4>+,tub-GAL80*^{ts} and *+>UAS-rpr* controls (p<0.0001 and p<0.0001,
538 respectively; Log-rank test); n=100-213 females.

539 (D) Lifespan was significantly longer in *dilp2-GAL4>UAS-rpr,tub-GAL80^{ts}* males compared
540 with *dilp2-GAL4>+,tub-GAL80^{ts}* and *+>UAS-rpr* controls (p<0.0001 and p<0.0001,
541 respectively; Log-rank test); n=100-148 males.

542 (E) The number of eggs laid by females in the first 24-hour period post-mating that
543 developed into pupae was significantly lower in *dilp2-GAL4>UAS-rpr,tub-GAL80^{ts}* females
544 compared with *dilp2-GAL4>+,tub-GAL80^{ts}* and *+>UAS-rpr* controls (p<0.0001 and
545 p<0.0001, respectively; one-way ANOVA followed by Tukey HSD). The number of eggs laid
546 by females in the first 24-hour period post-mating that developed into pupae produced by
547 females in the second (48 hr) and third (72 hr) 24-hour periods after mating was not
548 significantly different between *dilp2-GAL4>UAS-rpr,tub-GAL80^{ts}* females and *dilp2-*
549 *GAL4>+,tub-GAL80^{ts}* and *+>UAS-rpr* controls ([48 hours] p=0.5486 and p=0.3327; [72
550 hours] p=0.4374 and p=0.0142; Kruskal-Wallis test followed by Dunn's test). Total eggs laid
551 by females in the entire 72-hour period post-mating that developed into pupae produced
552 was significantly lower in *dilp2-GAL4>UAS-rpr,tub-GAL80^{ts}* females compared with *dilp2-*
553 *GAL4>+,tub-GAL80^{ts}* and *+>UAS-rpr* controls (p=0.0116 and p<0.0001, respectively; one-
554 way ANOVA followed by Tukey's HSD); n=30 females.

555 (F) The number of eggs laid by females mated to either *dilp2-GAL4>UAS-rpr,tub-GAL80^{ts}*
556 males or control *dilp2-GAL4>+,tub-GAL80^{ts}* and *+>UAS-rpr* males that developed into
557 pupae over the first (24 hr), second (48 hr), and third (72 hr) 24-hour periods after mating
558 was not significantly different ([24 hours] p=0.0032 and p=0.7420; [48 hours] p=0.0598 and
559 p=0.7339; [72 hours] p=0.0812 and p=0.7902; one-way ANOVA followed by Tukey's HSD).
560 The total number of eggs laid by females over a 72-hr period after mating with either *dilp2-*
561 *GAL4>UAS-rpr,tub-GAL80^{ts}* males or control *dilp2-GAL4>+,tub-GAL80^{ts}* and *+>UAS-rpr*
562 males that developed into pupae was not significantly different (p=0.0011 and p=0.9980,
563 respectively; one-way ANOVA followed by Tukey's HSD). n=22-30 males.

564 (G) Whole-body triglyceride levels were significantly lower in *dilp2-GAL4>UAS-rpr,tub-*
565 *GAL80^{ts}* females compared with *dilp2-GAL4>+,tub-GAL80^{ts}* and *+>UAS-rpr* control females
566 (p<0.0001 and p<0.0001, respectively). Whole-body triglyceride levels were not significantly
567 different between *dilp2-GAL4>UAS-rpr,tub-GAL80^{ts}* males and *dilp2-GAL4>+,tub-GAL80^{ts}*
568 and *+>UAS-rpr* control males (p=0.9397 and p=0.0022, respectively) (sex:genotype
569 interaction p=0.0002). Two-way ANOVA followed by Bonferroni post-hoc test; n=8 biological
570 replicates.

571 (H) Whole-body triglyceride levels were significantly lower in female *dilp2-GAL4>UAS-*
572 *rpr,tub-GAL80^{ts}* after heat shock compared with genotype-matched females without heat
573 shock (p<0.0001). A modest reduction in whole-body triglyceride levels was also observed
574 in males (p=0.0156); however, the magnitude of the decrease was smaller than in females
575 (sex:genotype interaction p=0.0258). Two-way ANOVA followed by Bonferroni post-hoc test;
576 n=16 biological replicates.

577 (I) Whole-body triglyceride levels were not significantly different between *dilp2-*
578 *GAL4>w^{1118},tub-GAL80^{ts}* females that received a heat shock compared with *dilp2-*
579 *GAL4>w^{1118},tub-GAL80^{ts}* females that received no heat shock (p=0.7266; Student's t-test);
580 n=8 biological replicates.

581 (J, K) Food consumption was not significantly different in either females (J) or males (K)
582 between *dilp2-GAL4>UAS-rpr,tub-GAL80^{ts}* flies and *dilp2-GAL4>+,tub-GAL80^{ts}* or *+>UAS-*
583 *rpr* controls (females: p=0.9999 [GAL4 control] and p=0.9318 [UAS control]; males:
584 p=0.6516 [GAL4 control] and p=0.0628 [UAS control]). One-way ANOVA followed by Tukey
585 HSD; n=8-9 biological replicates.

586 All data in (A, B, E-J) plotted as mean ± SEM. ns indicates not significant with p>0.05, *
587 p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

588 See also figure S3.

589 **Materials and methods**

590 **Fly strains**

591 The following fly strains from the Bloomington *Drosophila* Stock Center were used: *Canton-*
592 *S (CS)* (#64349), *w^{1118}* (#3605), *dilp2-GAL4* (IPCs) (#37516), *UAS-reaper (rpr)* (#5823),
593 *UAS-CaLexA* (*UAS-LexA-VP16-NFAT*) (#66542), *tubP-GAL80[ts]* (#7018). We obtained
594 *UAS-mRFP* as a kind gift from Dr Douglas Allan at The University of British Columbia.

595

596 **Fly husbandry**

597 The recipe for our regular lab diet (1S) is as follows: 20.5 g/L sucrose, 70.9 g/L D-glucose,
598 48.5 g/L cornmeal, 45.3 g/L yeast, 4.55 g/L agar, 0.5g CaCl2•2H2O, 0.5 g MgSO4•7H2O,
599 11.77 mL acid mix (propionic acid/phosphoric acid). To make 1 L of 20% food, 800 mL 0.7%
600 agar was mixed with 200 mL of 1S food. The low-sugar diet (0S) was made by leaving out
601 sucrose and D-glucose from the 1S diet. The 1S diet was calorie-matched to 0S (1S-low
602 calorie) by removing 8.6 g/L cornmeal from the 1S diet. For diets with sugar added back,
603 either 20.5 g/L sucrose (0S+sucrose), 70 g/L D-glucose (0S+D-glucose), or 62.5 g/L L-
604 glucose (0S+L-glucose; Biosynth, MG05247) were added to 0S. To make 30% high sugar
605 diet, 300 g/L extra sucrose was added to the 1S diet in line with published work [187,188]. For
606 ecdysone-supplemented food, 0.5 mg/mL of 20-hydroxyecdysone (20-HE) in ethanol was
607 added to the food (abcam, ab142425). For control food, the same ethanol volume without
608 20-HE was added to the food. For all experiments, we allowed female flies to lay eggs on
609 grape juice agar plates for 12 hr. At 24 hr after egg laying, 50 larvae were picked into vials
610 containing 10 mL food and reared at 22°C. Males and females were distinguished by the
611 presence of sex combs late in the pupal period and placed into single-sex vials to eclose.
612 After eclosion, adult flies were maintained at a density of twenty flies per vial in single-sex
613 groups. Unless otherwise stated, all experiments used 5- to 7-day-old virgin flies.

614

615 **Adult weight**

616 Groups of 10 flies were placed in preweighed 1.5 ml tubes (Diamed Lab Supplies,
617 DIATEC610-2550) and weighed on an analytical balance (Mettler-Toledo, ME104).

618

619 **Capillary feeder (CAFE) assay**

620 One biological replicate consisted of measurements from one group of 10 flies. Flies were
621 placed into a specialized 15 ml conical vial with access to two capillary tubes, as previously
622 described[80]. Briefly, capillary tubes were filled with fly food media containing nutrients
623 equivalent to the 1S diet (20.5 g/L sucrose, 70.9 g/L D-glucose, 45.3 g/L yeast extract in
624 water) or 0S diet (45.3 g/L yeast extract in water). To prevent evaporation, approximately
625 0.5 µl of mineral oil was added to the top of each capillary tube. The vials were placed into
626 fitted holes in the lid of a large plastic container. To maintain high humidity throughout the
627 experiment, a shallow layer of water was poured into the base of the container. The
628 meniscus of the fly food media was marked before the start of the experiment and again
629 after 24 hr. The distance between the marks was used to quantify the volume of fly food
630 media that was consumed by the 10 flies (1 mm=0.15 µl). To account for sex differences in
631 body size, the volume of fly food consumed was normalized to the weight of individual flies.

632

633 **Whole-body triglyceride measurements**

634 One biological replicate consisted of five flies. Flies were collected in a 1.5 ml tube and
635 homogenized in 200-350 µl of 0.1% Tween (Amresco, 0777-1L) in 1X phosphate-buffered
636 saline (PBS; Sigma-Aldrich, P5493) using 50 µl of glass beads (Sigma-Aldrich, Z250473)
637 agitated at 8 m/s for 5 s (OMNI International BeadRuptor 24). Triglyceride concentration was
638 measured using either Sigma kit (G7793, T2449, F6428) or Stanbio kit (FT7610, BD386a/d,
639 BD386b) according to the manufacturer's instructions and as described previously[4,5] with
640 the following modifications. For Sigma kit, 30 µl of homogenate was transferred in duplicate

641 to a 96-well plate and heat treated at 70°C for 10 min (Bio-Rad T100 Thermal Cycler).
642 Standard curve was prepared using glycerol standard (Sigma, G7793) for concentrations of
643 0 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, and 2 mg/ml. After heat treatment,
644 30 µl of each concentration of standard solution was transferred to the same 96-well plate
645 in duplicate. 30 µl of triglyceride reagent (Sigma, T2449) was added to 1 set of
646 samples/standard solutions and 0.1% Tween in 1x PBS was added to the other set of
647 samples/standard solutions. After 40 min incubation at 37°C, the 96-well plate was
648 centrifuged at 3800 rpm for 3 min at 4°C (VWR® Benchtop General Purpose Centrifuges,
649 10830-744) to precipitate cellular debris. 7.5 µl of supernatant was transferred to a 384-well
650 plate-reader plate and 25 µl of free glycerol reagent (Sigma, F6428) was added to each well.
651 Plate was incubated at 37°C for 5 min then absorbance was read at 540 nm (Thermo
652 Scientific – Multiskan FC Microplate Photometer). For Stanbio kit, 10 µl of either
653 homogenate or triglyceride standard (FT7610) was added to the 190 µl of activated
654 triglyceride reagent (Enzymatic Triglyceride Reagent, BD386a/d; Triglyceride Activator, Cat.
655 No. BD386b) in the 96-well plate. After 15 min incubation at room temperature, the
656 absorbance was read at 540 nm. Triglyceride concentration was used to calculate percent
657 body fat.
658
659 **Lipidomics and metabolomics**
660 **Sample preparation**
661 Abdominal carcasses were isolated from 5-day-old male and female w^{1118} flies maintained
662 on the 1S or 0S diet after eclosion. One biological replicate consisted of 30 abdominal
663 carcasses dissected from 5- to 7-day-old adult males and females; a total of 9 biological
664 replicates were collected for each sex and condition. After dissection, each carcass was
665 snap-frozen on dry ice in a 2 mL screw-cap microcentrifuge tube (Thermo Fisher Scientific,
666 Waltham, MA) and stored at –80˚C until further processing. For a dual-phase extraction,

667 samples were homogenized with a Mini-Beadbeater-24 (Biospec Products, Bartlesville,
668 United States) at 3800 rpm. A total of 333 µl 90% MeOH was added and two 15 sec
669 homogenization cycles were performed with a 1 min rest on dry ice in between. The
670 homogenized solution was mixed with 1000 µl of methyl tert-butyl ether and shaken for 5
671 min. To induce the phase separation, 317 µl of water was added. After mixing, the solution
672 rested at room temperature for 10 min and then the two-layer solution was centrifuged at
673 14,000 rpm for 15 min at 4°C. For LC-MS analysis, the clear upper and lower layers were
674 separated into new vials, dried in a SpeedVac at 20°C, and reconstituted in 70 µl of
675 acetonitrile and isopropanol (1:1, v/v) and 70 µl of acetonitrile and water (1:1, v/v) mixed
676 solvent, respectively. A method blank sample was also prepared following the same protocol
677 but without *Drosophila* abdomens.
678
679 **LC-MS/MS experiment**
680 For metabolomics, hydrophilic interaction chromatography (HILIC) separation was
681 performed on a SeQuant ZIC-HILIC column (50 mm × 2.1 mm, 5 µm, 200 Å) with an elution
682 time of 8 min. Mobile phase A was 5% acetonitrile in water with 10 mM ammonium acetate
683 (Thermo Fisher Scientific) with pH = 9.8, adjusted by ammonium hydroxide (MilliporeSigma),
684 and mobile phase B was 5% water in acetonitrile with no buffer. The LC elution gradient was
685 optimized using the BAGO software[189] as follows: 0 min, 10% A; 1 min, 10% A; 2 min, 20%
686 A; 5 min, 20% A; 6 min, 45% A; 7 min, 95% A; 8 min, 95% A. For lipidomics, reversed phase
687 (RP) separation was achieved on a Waters UPLC Acquity BEH C18 Column (1.0 mm ×100
688 mm, 1.7 µm, 130 Å, Milford, MA, USA) with an elution time of 25 min. Mobile phase A was
689 acetonitrile and water (6:4, v/v) with 2 mM ammonium formate (pH = 4.8, adjusted by formic
690 acid), and mobile phase B was isopropanol and acetonitrile (9:1, v/v). LC elution gradient
691 was optimized using the BAGO software as follows: 0 min, 5% B; 1 min, 5% B; 4 min, 35%

692 B; 7 min, 40% B; 10 min, 50% B; 13 min, 55% B; 16 min, 55% B; 19 min, 75% B; 22 min,
693 75% B; 25 min, 95% B.
694 The MS was operated in electrospray ionization (ESI) positive mode. The ESI source
695 conditions were set as follows: dry gas temperature, 220°C; dry gas flow, 7 L/min; nebulizer
696 gas pressure, 1.6 bar; capillary voltage, 4500 V for positive mode. The MS1 analysis was
697 conducted using following parameters: mass range, 70-1000 *m/z*; spectrum type: centroid,
698 calculated using maximum intensity; absolute intensity threshold: 250. Data-dependent
699 MS/MS analysis parameters: collision energy: 16-30 eV; cycle time: 3 s; spectra rate: 4 Hz
700 when intensity < 10^4 and 12 Hz when intensity > 10^5, linearly increased from 10^4 to 10^5.
701 External calibration was applied using sodium formate to ensure the *m/z* accuracy before
702 sample analysis.
703
704 **Data processing**
705 The raw MS data were converted to ABF format in Reifycs Abf Converter (ver. 4.0.0). The
706 converted data were processed in MS-DIAL (ver. 4.90) for feature extraction, feature
707 alignment, and compound identification. The data processing parameters in MS-DIAL were
708 set as follows: MS1 tolerance, 0.01 Da; MS/MS tolerance, 0.05 Da; mass slice width, 0.05
709 Da; smoothing method, linear weighted moving average; smoothing level, 3 scans; minimum
710 peak width, 5 scans. Compound identification was achieved using NIST 20 for metabolomics
711 and the internal library within MS-DIAL for lipidomics. Post-acquisition sample normalization
712 was performed using the *MAFFIN* R package[190] to ensure fair quantitative comparison.
713
714 **Excrement glucose assay**
715 One biological replicate consists of 30 female flies or 60 male flies. Flies were fed with 1S
716 diet containing 5% blue dye (FD&C Blue no.1, Ward's Science, 470300-996) for 3.5 hr, and
717 subsequently transferred into a 1.5 mL tube for excrement collection at room temperature.

718 After 6 hr in the 1.5 mL tube, flies were discarded and 100 µL of 1x PBS was added to each
719 tube; tubes were vortexed to dissolve and pool all the blue-stained excrement deposited in
720 the tube. For blue dye measurement, 10 µL of the excrement was added to 10 µL of 1x PBS
721 in 384-well plate and absorbance was measured at 630 nm (Thermo Scientific – Multiskan
722 FC Microplate Photometer). The rest of the excreta (90 µL) was added to 110 µL of Glucose
723 Reagent (Sigma-Aldrich, GAHK20) in 96-well plate and incubated overnight at 37 °C for
724 measurement of glucose levels at 340 nm (Tecan Austria GmbH-Tecan Spark). Glucose
725 content in the excrement was normalized with blue dye to account for sex differences in
726 excreta volume, and to account for the number of flies that deposited the excreta[191].
727
728 **RNA extraction, cDNA synthesis, and Quantitative real-time PCR (qPCR)**
729 One biological replicate consisted of 10 adult heads or 15 adult fat bodies. Samples were
730 homogenized in 500 µl of Trizol (Thermo Fisher Scientific; 15596018) and precipitated using
731 isopropanol and 75% ethanol to extract RNA. Pelleted RNA was resuspended using 20-30
732 µl of molecular biology grade water (Corning, 46-000-CV) and stored at -80°C until use.
733 Each experiment contained 4-7 biological replicates per sex, per genotype, and per diet;
734 each experiment was repeated twice.
735 For cDNA synthesis, an equal amount of RNA per reaction was DNase-treated and
736 reverse transcribed according to manufacturer's instructions using the QuantiTect Reverse
737 Transcription Kit (Qiagen, 205314). Relative mRNA transcript levels were quantified using
738 qPCR as described previously[5]. Data were normalized to the average fold change of *Actin5C*
739 and *β-tubulin*. For a full primer list, refer to Supplemental Table S1.
740
741 **IPC activity measurement**
742 To isolate the Dilp-producing cells (IPC), individual flies were removed from food and
743 anesthetized briefly with CO_2. Brains were dissected in cold 1× PBS. Samples were fixed in

744 4% paraformaldehyde (Electron Microscopy Science 15710) for 40 min, followed by two
745 washes in cold 1× PBS. Samples were incubated with Hoechst (Sigma-Aldrich, 33342) at a
746 concentration of 1:1000 for 30 min and mounted in 75% (w/v) sucrose solution. Images were
747 captured using a LSI3-Leica SP5 laser scanning confocal microscope and processed using
748 Fiji (ImageJ;[192]). To quantify IPC activity we used IPC-specific GAL4 driver *dilp2-GAL4*[99] to
749 drive expression of both *UAS-mRFP* and *UAS-CaLexA* (*UAS-LexA-VP16-NFAT*)[86]. GFP
750 intensity in each IPC cluster was quantified by measuring the sum of pixel intensity within
751 the region of interest for both GFP and RFP. GFP was normalized to RFP that was co-
752 expressed in the APC to account for inter-individual and/or sex differences in GAL4-driven
753 transgene expression (ImageJ;[192]).
754
755 **Insulin sensitivity test**
756 To measure insulin sensitivity, we adapted a published protocol[77]. Briefly, abdominal
757 carcasses were isolated from 5-day-old adult flies; one biological replicate consisted of 15
758 abdominal carcasses. The carcasses were placed in a dish with ice-cold Schneider's
759 *Drosophila* solution (21720024, Thermo Scientific) for 30 min. After this initial incubation,
760 Schneider's media was replaced with 2 mL of fresh Schneider's *Drosophila* solution or fresh
761 Schneider's *Drosophila* solution with recombinant human insulin (final concentration 1 μM;
762 I2643, Sigma-Aldrich) and incubated at room temperature for 30 min. After stimulation,
763 carcasses were collected in a tube, snap-frozen, and used for RNA isolation and qPCR
764 analysis.
765
766 **Lifespan**
767 After eclosion, unmated male and female flies were transferred to new vials of fresh food
768 every 2–3 days until no living flies remained in the vial. Deaths were recorded when the flies
769 were transferred to fresh food.

770 **Female fecundity**

771 One 5-day-old virgin female was placed with a group of three 5-day-old virgin *CS* males for
772 1 hr. After a 1 hr mating period, the males were removed and the females were allowed to
773 lay eggs. Females were transferred into new vials for egg-laying every 24 hr; the total
774 number of pupae in each vial was counted several days later.

775

776 **Male fertility**

777 One singly-housed 5-day-old virgin male was placed with a group of three 5-day-old virgin
778 *CS* females and allowed to interact for 1 hr. After the 1 hr mating period, the male was
779 removed from the vial and the females were allowed to lay eggs. Females were transferred
780 into new vials for egg-laying every 24 hr; the total number of pupae in each vial was counted
781 several days later.

782

783 **Statistical analysis**

784 Statistical analyses and data presentation were completed using GraphPad Prism 9
785 (GraphPad Software, San Diego, CA, USA) (Supplemental Table S2). All data were tested
786 for normality using the Shapiro-Wilk test. Normally-distributed data was subjected to
787 parametric tests, including Student's *t*-test, one-way ANOVA followed by Tukey's HSD, and
788 two-way ANOVA followed by Bonferroni post-hoc test as appropriate. For non-normally-
789 distributed data we used Mann-Whitney test and Kruskal-Wallis test followed by Dunn's test.
790 For all statistical analyses, differences were considered significant if the $p<0.05$.

791 **Supplemental figure legends**

792 **Figure S1. Dietary sugar levels change body fat, but not food consumption, in adult**
793 **flies. Related to Figure 1.**

794 (A) There was a trend toward higher food consumption in 5-day-old adult w^{1118} females
795 compared with age-matched males (p=0.0812; Student's t-test); n=11 biological replicates.

796 (B) Whole-body triglyceride levels were significantly lower in 5-day-old *CS* females
797 maintained on the 0S diet compared with females kept on the 1S diet (p=0.0181); no
798 significant change in body fat was observed in males (p=0.0582; sex:diet interaction
799 p=0.7239). Two-way ANOVA followed by Bonferroni post-hoc test; n=8 biological replicates.

800 (C) Food consumption was not significantly different between 5-day-old adult w^{1118} females
801 and males maintained on the 1S diet compared with sex-matched flies kept on the 0S diet
802 (females: p>0.9999; males: p>0.9999; sex:diet interaction p=0.5654). Two-way ANOVA
803 followed by Bonferroni post-hoc test; n=10-11 biological replicates. Note: food consumption
804 data in 1S diet was replotted from panel S1A as food intake data on 1S and 0S were
805 collected in the same series of experiments.

806 (D) Whole-body triglyceride levels were significantly lower in 5-day-old w^{1118} adult females
807 and males maintained on 0S with L-glucose diet compared with sex-matched flies kept on
808 the 1S diet (females: p<0.0001; males: p<0.0001); the magnitude of the decrease in body
809 fat was greater in females than in males (sex:diet interaction p=0.0029). Two-way ANOVA
810 followed by Bonferroni post-hoc test; n=8 biological replicates. Note: the 1S triglyceride data
811 was replotted from Figure 1E as they were part of the same large experiment.

812 All data plotted as mean ± SEM. ns indicates not significant with p>0.05; * p<0.05, ** p<0.01,
813 *** p<0.001, **** p<0.0001.

814 **Figure S2. Sugar-induced changes in lipid species in the adult fat body. Related to**
815 **Figure 2.**

816 (A) Sex bias of fatty acids in the triglyceride pool with significant differences in abundance
817 between female and male fat bodies in 1S diet ($p<0.01$; Student's t-test).

818 (B) Proportion of species in each lipid subclass that are differentially regulated between the
819 fat body of females maintained on the 1S and females kept on the 0S diet ($p<0.01$; Student's
820 t-test). Numbers on histograms indicate the number of species with differential abundance.

821 (C, D) Triglyceride species with significant changes in abundance between females
822 maintained on a 1S diet compared with females kept on the 0S diet ($p<0.01$; Student's t-
823 test); species organized by number of carbons C35-C48 (C) and C49-C62 (D).

824 (E) Fatty acids in the triglyceride pool with significant differences in abundance between
825 females maintained on either the 1S or the 0S diet ($p<0.01$; Student's t-test).

826 (F) Triglyceride species with significant changes in abundance between males maintained
827 on a 1S diet compared with males kept on the 0S diet ($p<0.01$; Student's t-test).

828 (G) Proportion of species in each lipid subclass that are significantly different in abundance
829 between the fat bodies of males raised on the 1S or the 0S diet ($p<0.01$; Student's t-test).
830 Numbers on histograms indicate the number of species with differential abundance.

831 TG: triglyceride; TG-O: ether-linked TG; DG: diglyceride; PC: phosphatidylcholine; PC-O:
832 ether-linked PC; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI:
833 phosphatidylinositol;. Acyl-Car: acyl-carnitine; Cer: Ceramide.

834 **Figure S3. Ecdysone feeding does not increase body fat in females. Related to Figure**
835 **4**.

836 (A) Whole-body triglyceride levels were significantly lower in 5-day-old adult w^{1118} females
837 maintained on a diet supplemented with 0.5 mg/mL 20-HE compared with control females
838 (p=0.0067, Student's t-test); n=8 biological replicates.

839 Data plotted as mean ± SEM. ** p<0.01.

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Figure 1

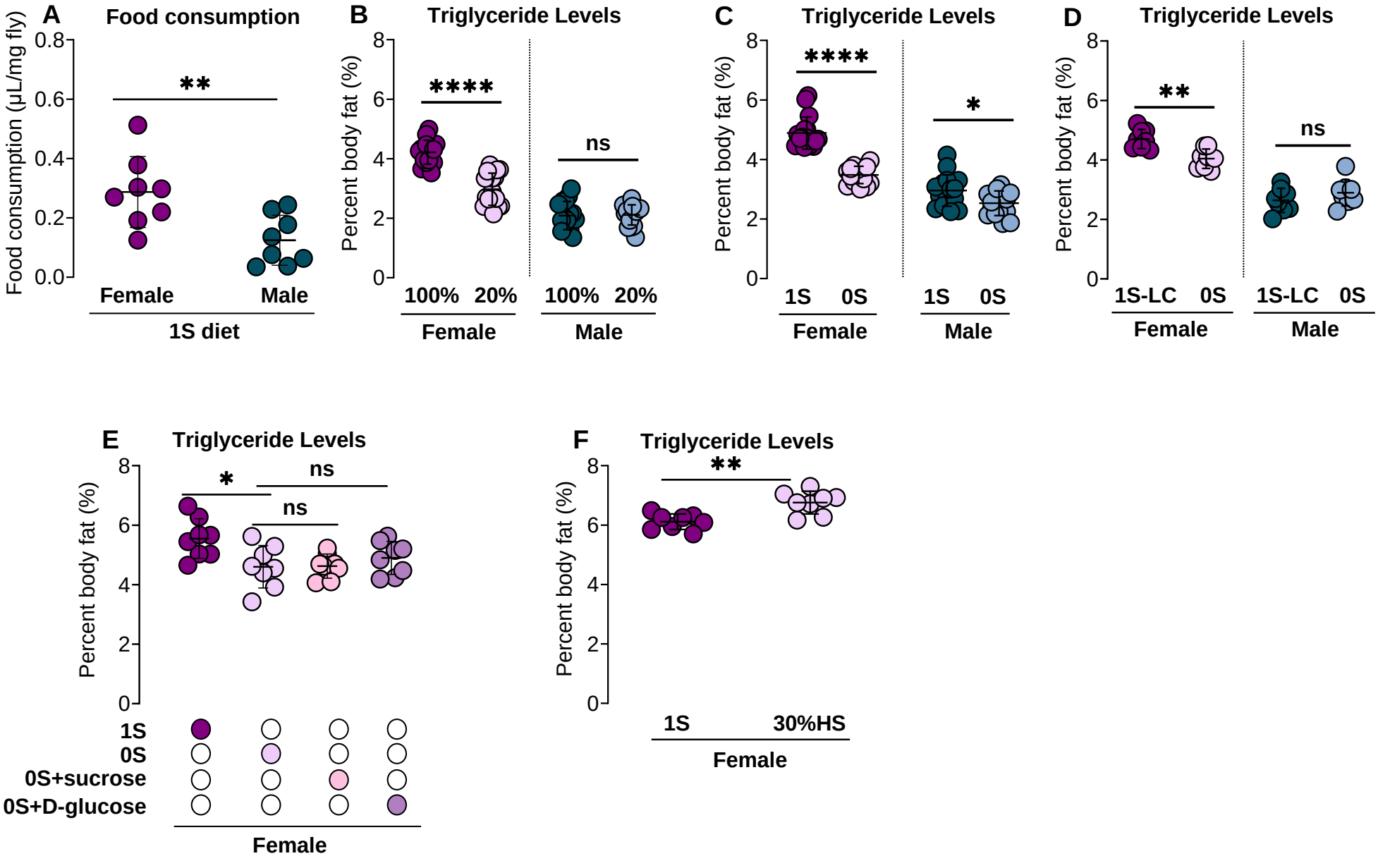

A
Food consumption
Food consumption (µL/mg fly)
0.8
0.6
0.4
0.2
0.0
**
Female
Male
1S diet
B
Triglyceride Levels
Percent body fat (%)
8
6
4
2
0
****
ns
100%
20%
100%
20%
Female
Male
C
Triglyceride Levels
Percent body fat (%)
****
*
1S
0S
1S
0S
Female
Male
D
Triglyceride Levels
Percent body fat (%)
**
ns
1S-LC
0S
1S-LC
0S
Female
Male
E
Triglyceride Levels
Percent body fat (%)
*
ns
ns
1S
0S
0S+sucrose
0S+D-glucose
Female
F
Triglyceride Levels
Percent body fat (%)
**
1S
30%HS
Female

Figure 2

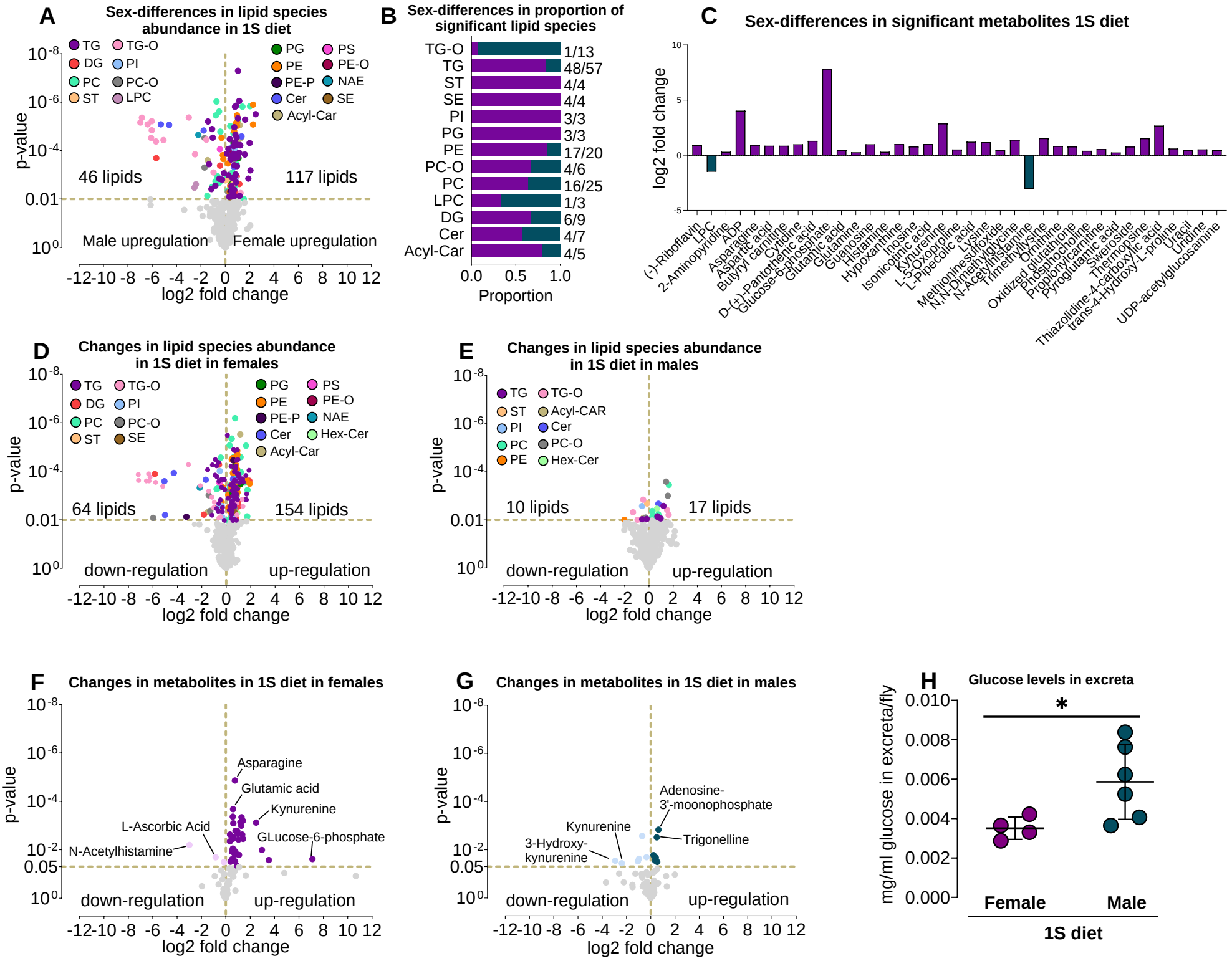

A
Sex-differences in lipid species abundance in 1S diet
TG
TG-O
DG
PI
PC
PC-O
ST
LPC
PG
PS
PE
PE-O
PE-P
NAE
Cer
SE
Acyl-Car
p-value
46 lipids
117 lipids
Male upregulation
Female upregulation
log2 fold change
B
Sex-differences in proportion of significant lipid species
TG-O 1/13
TG 48/57
ST 4/4
SE 4/4
PI 3/3
PG 3/3
PE 17/20
PC-O 4/6
PC 16/25
LPC 1/3
DG 6/9
Cer 4/7
Acyl-Car 4/5
Proportion
C
Sex-differences in significant metabolites 1S diet
log2 fold change
(-)-Riboflavin
LPC
2-Aminopyridine
ADP
Asparagine
Aspartic acid
Butyryl carnitine
Cytidine
D-(+)-Pantothenic acid
Glucose-6-phosphate
Glutamic acid
Glutamine
Guanosine
Histamine
Hypoxanthine
Inosine
Isonicotinic acid
Kynurenine
L-5-Oxoproline
L-Pipecolic acid
Lysine
Methionine sulfoxide
N,N-Dimethylglycine
N-Acetylhistamine
Trimethylysine
Ornithine
Oxidized glutathione
Phosphocholine
Propionylcarnitine
Pyroglutamic acid
Sweroside
Thermopsine
Thiazolidine-4-carboxylic acid
trans-4-Hydroxy-L-proline
Uracil
Uridine
UDP-acetylglucosamine
D
Changes in lipid species abundance in 1S diet in females
TG
TG-O
DG
PI
PC
PC-O
ST
SE
PG
PS
PE
PE-O
PE-P
NAE
Cer
Hex-Cer
Acyl-Car
p-value
64 lipids
154 lipids
down-regulation
up-regulation
log2 fold change
E
Changes in lipid species abundance in 1S diet in males
TG
TG-O
ST
Acyl-CAR
PI
Cer
PC
PC-O
PE
Hex-Cer
p-value
10 lipids
17 lipids
down-regulation
up-regulation
log2 fold change
F
Changes in metabolites in 1S diet in females
p-value
Asparagine
Glutamic acid
Kynurenine
L-Ascorbic Acid
GLucose-6-phosphate
N-Acetylhistamine
down-regulation
up-regulation
log2 fold change
G
Changes in metabolites in 1S diet in males
p-value
Adenosine-3'-moonophosphate
Kynurenine
Trigonelline
3-Hydroxy-kynurenine
down-regulation
up-regulation
log2 fold change
H
Glucose levels in excreta
mg/ml glucose in excreta/fly
*
Female
Male
1S diet

Figure 3

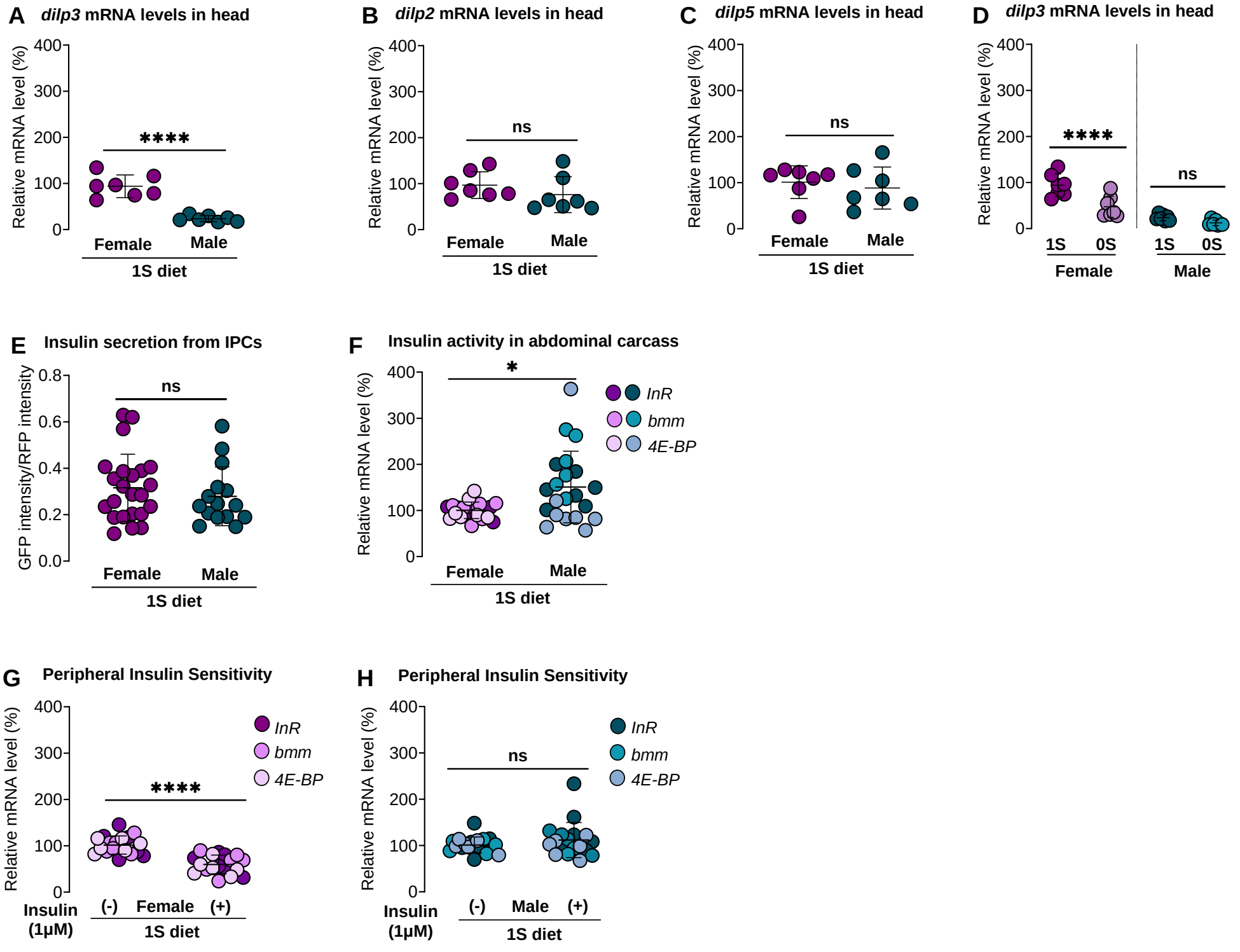
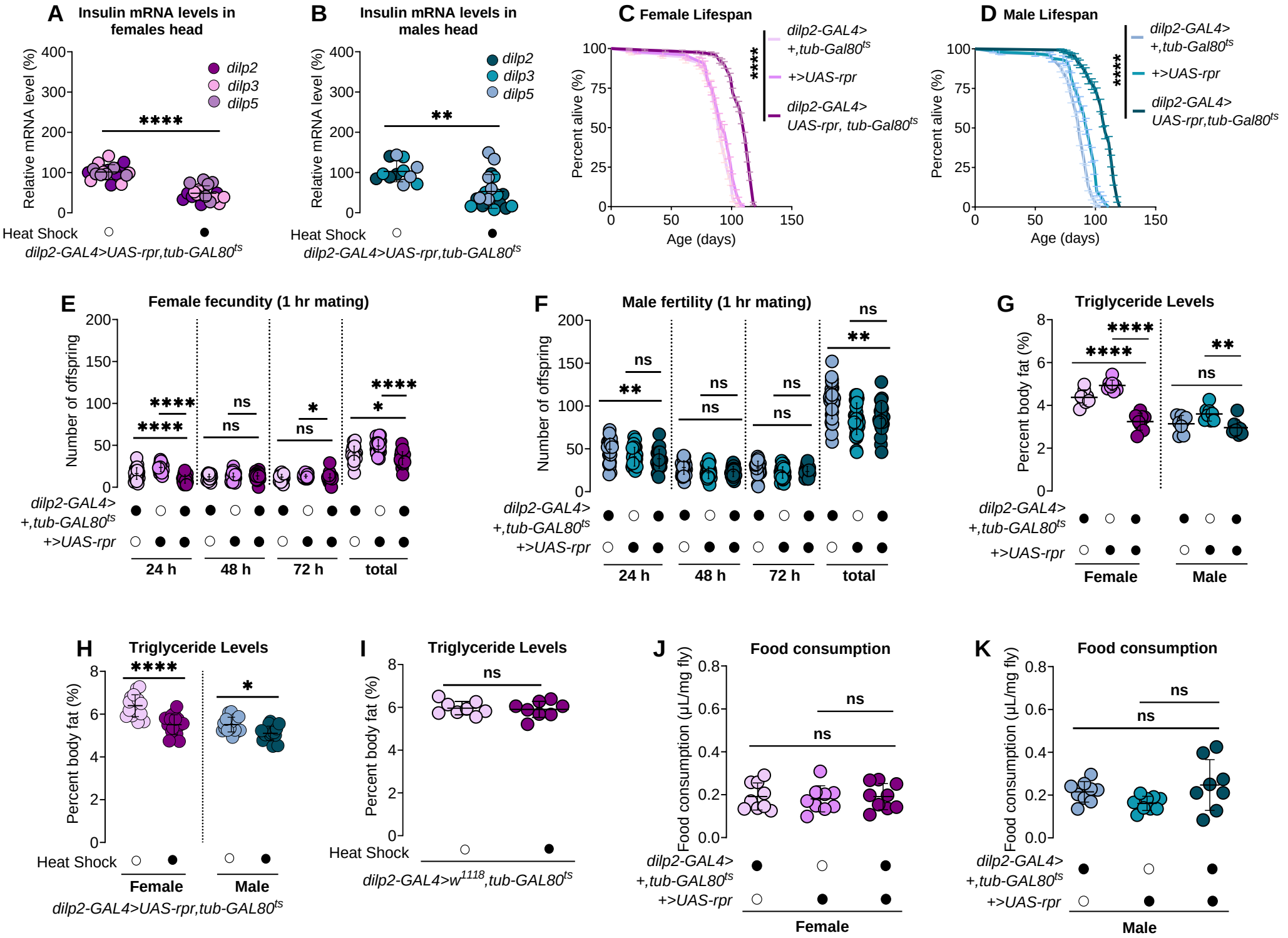
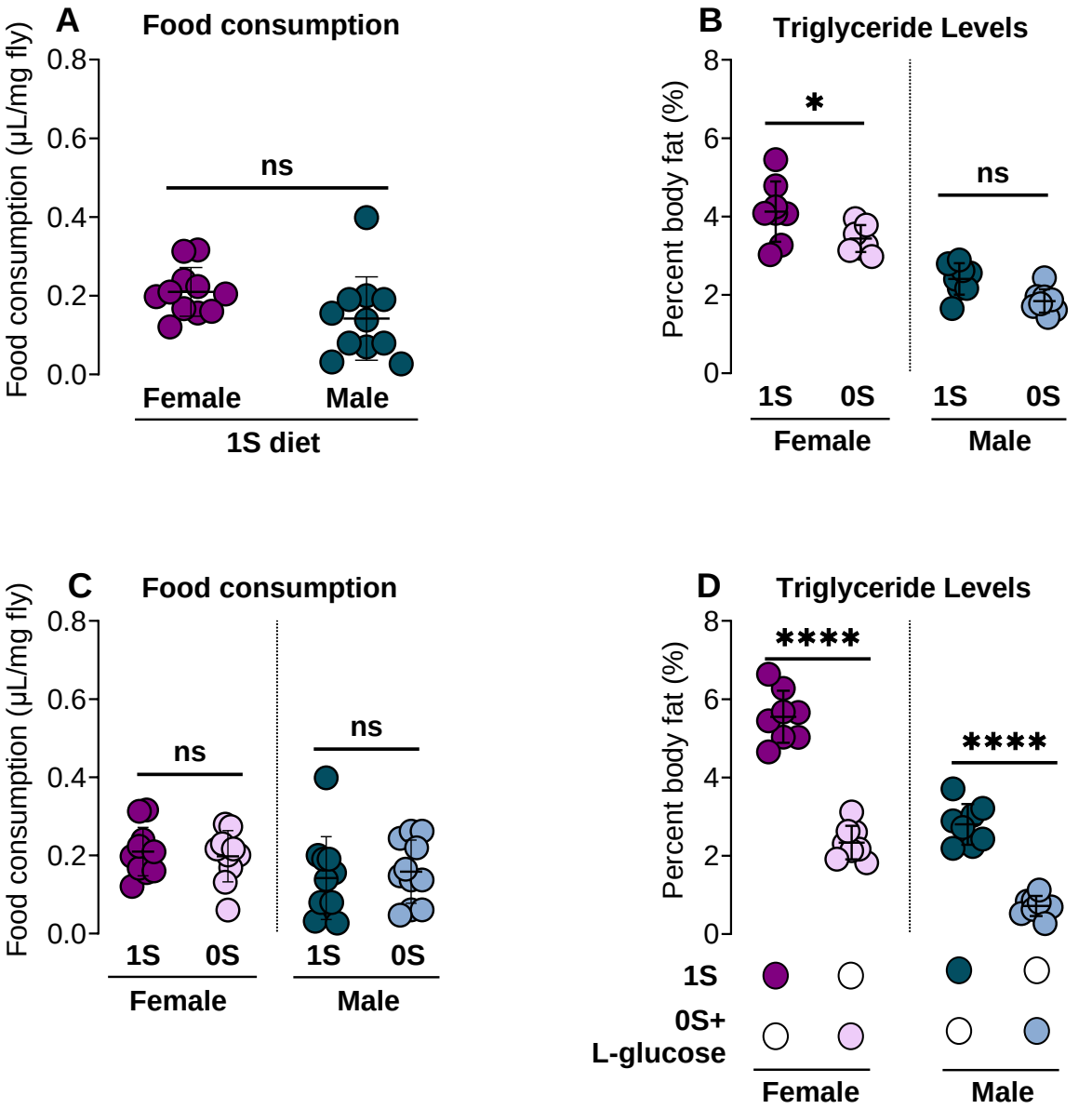

A
dilp3 mRNA levels in head
Relative mRNA level (%)
400
300
200
100
0
****
Female
Male
1S diet
B
dilp2 mRNA levels in head
Relative mRNA level (%)
ns
Female
Male
1S diet
C
dilp5 mRNA levels in head
Relative mRNA level (%)
ns
Female
Male
1S diet
D
dilp3 mRNA levels in head
Relative mRNA level (%)
****
ns
1S
0S
1S
0S
Female
Male
E
Insulin secretion from IPCs
GFP intensity/RFP intensity
0.8
0.6
0.4
0.2
0.0
ns
Female
Male
1S diet
F
Insulin activity in abdominal carcass
Relative mRNA level (%)
*
InR
bmm
4E-BP
Female
Male
1S diet
G
Peripheral Insulin Sensitivity
Relative mRNA level (%)
InR
bmm
4E-BP
****
Insulin (1μM)
(-)
Female
(+)
1S diet
H
Peripheral Insulin Sensitivity
Relative mRNA level (%)
InR
bmm
4E-BP
ns
Insulin (1μM)
(-)
Male
(+)
1S diet

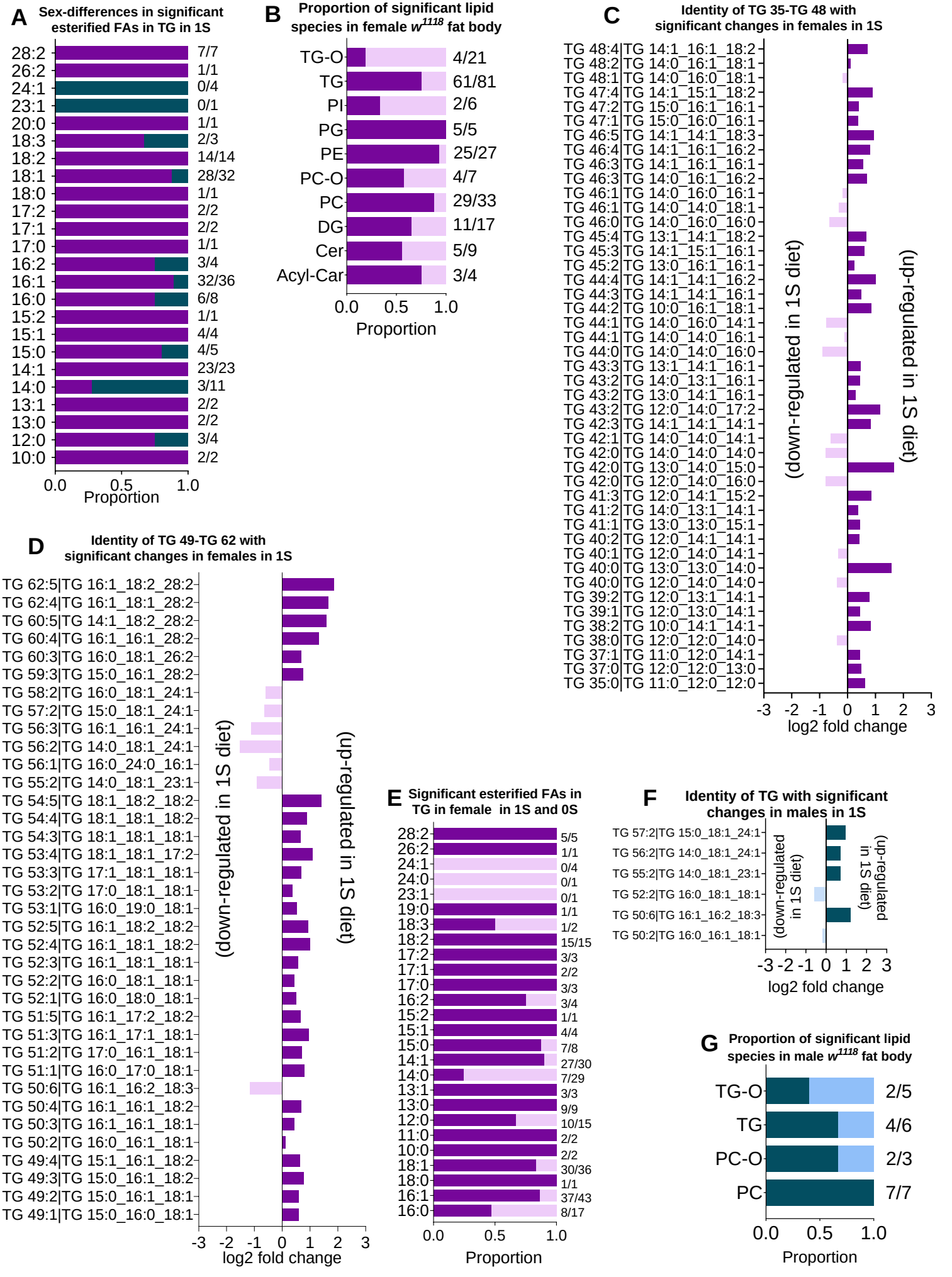
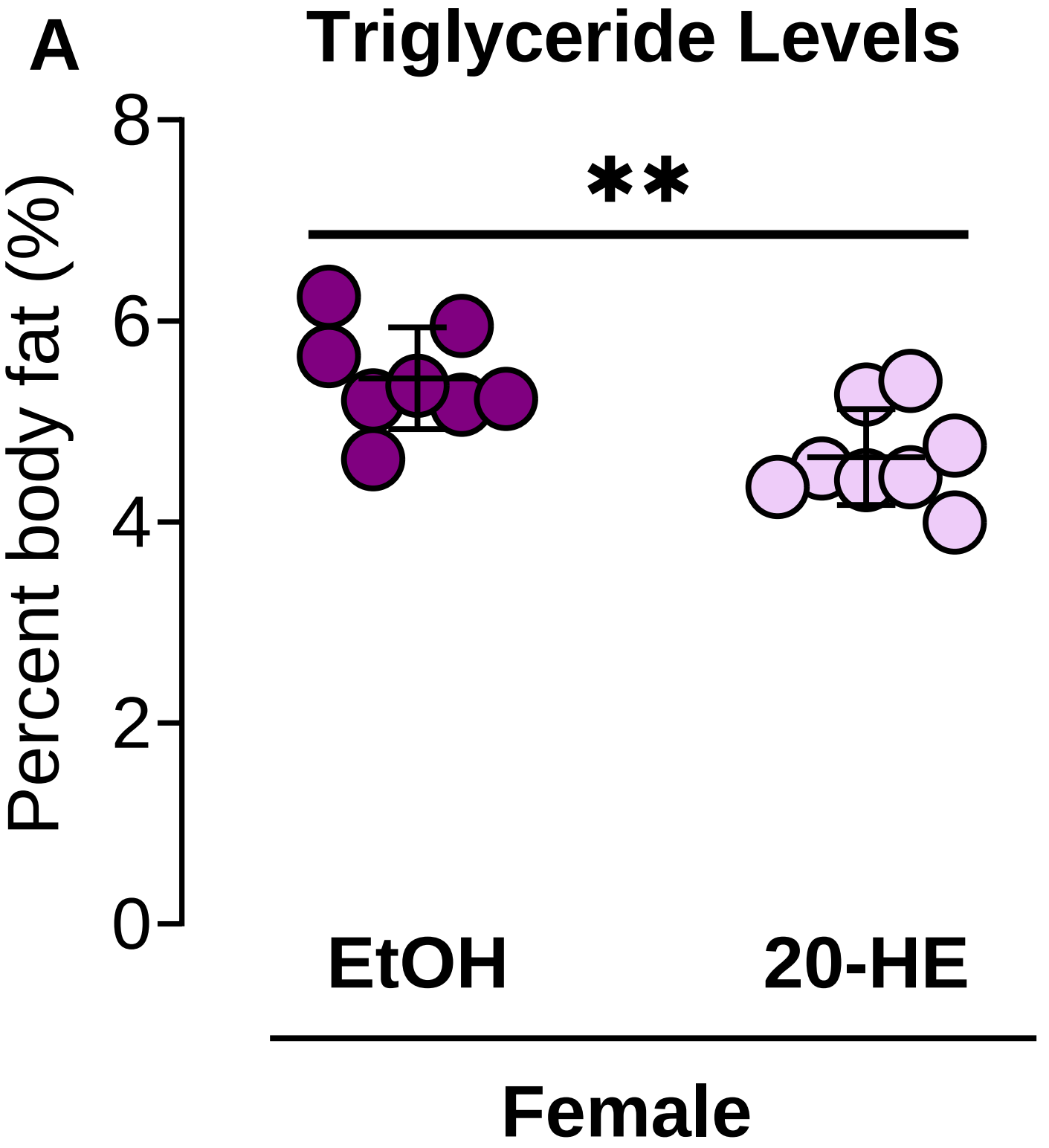
Figure 4


A
Insulin mRNA levels in females head
Relative mRNA level (%)
dilp2
dilp3
dilp5
****
Heat Shock
dilp2-GAL4>UAS-rpr,tub-GAL80ts
B
Insulin mRNA levels in males head
Relative mRNA level (%)
dilp2
dilp3
dilp5
**
Heat Shock
dilp2-GAL4>UAS-rpr,tub-GAL80ts
C
Female Lifespan
Percent alive (%)
Age (days)
****
dilp2-GAL4> +,tub-Gal80ts
+>UAS-rpr
dilp2-GAL4> UAS-rpr, tub-Gal80ts
D
Male Lifespan
Percent alive (%)
Age (days)
****
dilp2-GAL4> +,tub-Gal80ts
+>UAS-rpr
dilp2-GAL4> UAS-rpr,tub-Gal80ts
E
Female fecundity (1 hr mating)
Number of offspring
dilp2-GAL4> +,tub-GAL80ts
+>UAS-rpr
24 h
48 h
72 h
total
F
Male fertility (1 hr mating)
Number of offspring
dilp2-GAL4> +,tub-GAL80ts
+>UAS-rpr
24 h
48 h
72 h
total
G
Triglyceride Levels
Percent body fat (%)
dilp2-GAL4> +,tub-GAL80ts
+>UAS-rpr
Female
Male
H
Triglyceride Levels
Percent body fat (%)
Heat Shock
Female
Male
dilp2-GAL4>UAS-rpr,tub-GAL80ts
I
Triglyceride Levels
Percent body fat (%)
Heat Shock
dilp2-GAL4>w1118,tub-GAL80ts
J
Food consumption
Food consumption (µL/mg fly)
dilp2-GAL4> +,tub-GAL80ts
+>UAS-rpr
Female
K
Food consumption
Food consumption (µL/mg fly)
dilp2-GAL4> +,tub-GAL80ts
+>UAS-rpr
Male

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3