1 **Title**

2 Sex-biased expression of enteroendocrine cell-derived hormones contributes to higher

3 fat storage in *Drosophila* females

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Abstract

Enteroendocrine (EE) cells in the *Drosophila* gut produce and release multiple factors, including Allatostatin A (AstA), Allatostatin C (AstC), neuropeptide F (NPF), tachykinin (Tk), Diuretic hormone 31 (Dh31), Bursicon, CCHamide 1 (CCHa1) and CCHamide 2 (CCHa2), and short neuropeptide F (sNPF). Collectively, these peptides ensure that physiology (e.g., fat storage, fluid balance) and behavior (e.g., feeding, sleep) are coordinated with environmental factors such as nutrient quantity and quality. Despite notable sex differences in physiology and behavior, it remains unclear whether the regulation and function of these EE cell-derived factors is shared between males and females. Given that recent data identified sex-biased physiological effects of two EE cell-derived hormones on *Drosophila* food intake and energy mobilization, we performed a detailed characterization of these hormones in male and female flies. Despite an overall male bias in mRNA levels of *AstA*, *AstC*, *Tk*, *NPF*, *Dh31* in the whole body and head, we observed a strong female bias in mRNA levels of *AstC*, *Tk*, and *NPF* in the gut. To determine whether this sex-biased regulation was physiologically significant, we monitored triglyceride levels in flies with gut-specific loss of EE cell-derived hormones. In 5-day-old flies, loss of either EE cell-derived AstC or Tk reduced fat storage in females with no effect in males. These female-specific effects on fat storage were reproduced in flies with neuron-specific loss of the AstC (AstC-R2) and Tk receptors (TkR99D). Together, these data uncover strongly sex-biased regulation of EE cell-derived hormones, and show that gut-specific loss of two of these hormones had a female-specific effect on body fat.

Keywords

Sex difference, gut, enteroendocrine cell, physiology, triglyceride, hormones, fat storage

Highlights:

- Enteroendocrine cell-expressed hormones show strongly sex-biased expression
- Loss of enteroendocrine cell-derived AstC and Tk reduced body fat only in females
- Neuronal loss of AstC or Tk receptors reduced stored fat only in females

43 **1. Introduction**

44 In *Drosophila*, females store more fat than males [1–7]. Greater female fat storage has
45 been observed in both mated and unmated females compared with age-matched males
46 [1,3–7]. In flies, as in other animals, triglyceride is the main form of stored fat. Although
47 triglyceride is present in many cell types and organs (*e.g.*, oenocytes, glia, neurons, gut)
48 [5,6,8–14], the majority of triglyceride is stored in an organ called the fat body [3,12,15].
49 In females, high levels of triglyceride in the fat body play a key role in supporting
50 reproduction and physiology [5,16]. Indeed, triglyceride from the fat body is a key
51 energy source for the developing embryo [17]. Increased fat storage in adult females
52 also supports prolonged survival during nutrient deprivation compared with males [4,6].
53 Despite these clear benefits of greater fat storage for female fertility, excess fat
54 accumulation in males adversely affects their reproductive output. Specifically, males
55 carrying a mutation that promotes excess triglyceride accumulation show reduced testis
56 size, defects consistent with delays in spermatogenesis, and ultimately a reduction in
57 sperm number [8]. The sex difference in fat storage therefore likely reflects the fact that
58 males and females differ in how much whole-body triglyceride storage supports optimal
59 fertility.
60 Recent studies have identified genes and pathways that contribute to sex
61 differences in fat storage [5–7,18]. In mated females, the steroid hormone ecdysone
62 acts on neurons to promote food intake, which is associated with increased body fat [5].
63 In unmated adult females, the insulin/insulin-like growth factor signaling pathway (IIS)
64 plays a key role in maintaining an elevated level of triglyceride storage compared with
65 adult males [18]. Specifically, adult females have higher production of *Drosophila*

66 insulin-like peptide 3 (Dilp3) and greater insulin sensitivity, leading to higher IIS activity.
67 This elevated IIS activity is important for females to store more triglyceride than males,
68 as adult-specific ablation of the insulin-producing cells reduces body fat in females but
69 not males [18].

70 In males, body fat is maintained via alternative mechanisms. For example, males
71 show higher expression and activity of two catabolic pathways that promote fat
72 breakdown. One pathway is regulated by triglyceride lipase *brummer* (*bmm*), where
73 males show higher *bmm* mRNA levels compared with females [6]. This elevated *bmm*
74 expression contributes to the sex difference in fat storage by restricting triglyceride
75 accumulation in males. Similarly, males have higher production and secretion of
76 Adipokinetic hormone (Akh), a key lipolytic hormone in insects [7]. As with *bmm*, high
77 levels of Akh in males contribute to the sex difference in fat storage by limiting fat
78 accumulation in males [7]. Together, these studies have defined a model of the sex
79 difference in fat storage in which females maintain higher levels of fat storage due to the
80 anabolic action of IIS, whereas males have lower fat storage due to the catabolic effects
81 of *bmm* and Akh. While some progress has been made in revealing the mechanisms
82 underlying the sex-specific regulation of Akh and IIS [7,18], we do not have a complete
83 understanding of the factors that determine the sex-biased regulation and function of
84 these key metabolic factors.

85 Recent clues into the regulation of Akh, *bmm*, and IIS have emerged from
86 studies on peptide hormone function [19–30], as several hormones influence physiology
87 via effects on Akh- and Dilp-producing cells [19–22,24–33]. In particular, recent studies
88 have illuminated an important role for hormones produced by the enteroendocrine (EE)

89 cells of the gut [32,34–36]. Adult *Drosophila* EE cells produce and release hormones
90 such as Allatostatin A (AstA), Allatostatin C (AstC), neuropeptide F (NPF), tachykinin
91 (Tk), Diuretic hormone 31 (Dh31), Bursicon, CCHamide 1 (CCHa1) and CCHamide 2
92 (CCHa2), and short neuropeptide F (sNPF) [37–41].

93 EE cells are identified by expression of the homeodomain protein Prospero in
94 adults [42,43], where EE cells that produce distinct hormones are present in
95 anatomically defined regions of the adult gut [43]. For example, AstA- and Dh31-
96 producing EE cells are located in the posterior midgut, whereas EE cells that produce
97 AstC and Tk are found along the entire length of the midgut [37]. NPF-producing cells
98 are found in the anterior and middle midgut [41]. Supporting a role for EE cell-derived
99 hormones in regulating Akh/IIS, in fed conditions studies show EE cell-derived
100 hormones such as Bursicon inhibit Akh secretion [28], whereas NPF enhances Dilp
101 secretion from the insulin-producing cells [29]. During starvation, AstC promotes Akh
102 release from Akh-producing cells to enable lipid mobilization [22]. While at least two EE
103 cell-derived hormones have been shown to have sex-biased effects on food intake and
104 energy mobilization [22,44], sex differences in the regulation and function of most of
105 these hormones remain unclear. Defining potential differences in EE cells is an
106 important task, as prior studies have revealed profound differences in gut biology
107 between males and females.

108 For example, males and females differ in overall gut size and shape [45,46] and
109 in the number of intestinal stem cell divisions [45,47–49]. The absorptive lining of the
110 gut also shows sex differences during aging [48,50]. After mating, females show
111 pronounced changes to gut size, function, and gene expression [45,51–54]. While sex

112 differences in intestinal stem cells and enterocytes play a key role in mediating these
113 differences in gut biology, we know less about male-female differences in EE cells. We
114 therefore aimed to perform a detailed characterization of EE cell-derived hormones in
115 males and females, and to determine the contribution of these hormones to physiology
116 in each sex. We reveal profound sex-biased regulation of EE cell-expressed hormones
117 AstC, Tk, and NPF: females show higher mRNA levels of these hormones in the gut
118 than males. For at least two EE cell-derived hormones this sex-biased regulation was
119 physiologically significant, as loss of AstC and Tk in the gut reduced fat storage in
120 females with no effect in males. These female-specific fat storage defects were
121 reproduced by loss of AstC and Tk receptors in neurons and/or neuropeptide-producing
122 cells. While the specific cell type targeted by these EE cell-derived hormones to
123 influence fat storage remains unclear, this reveals a female-specific contribution of EE
124 cell-derived hormones in regulating body fat.
125

126 **2. RESULTS**

127 **2.1. Sex differences in expression of gut-derived peptide hormones**

128 Given that several EE cell-derived hormones such as AstA (FBgn0015591), AstC
129 (FBgn0032336), Tk (FBgn0037976), NPF (FBgn0027109), Dh31 (FBgn0032048) are
130 expressed in cells outside the gut [20,55–69], we used quantitative real-time PCR
131 (qPCR) to assess whole-body mRNA levels of genes encoding EE cell-expressed
132 hormones. In particular, we focused on hormones known to influence whole-body fat
133 metabolism [20,22,29,44,70,71]. In 5-day-old w^{1118} unmated adult males and females,
134 we found that whole-body mRNA levels of *AstA*, *AstC*, *Tk*, *NPF*, and *Dh31* were

135 significantly higher in males than in females (Figure 1A-E). To gain further insight into
136 this sex-biased expression, we analyzed mRNA levels of these factors from isolated
137 heads and intestines, as these are the main sites of AstA, AstC, Tk, NPF, and Dh31
138 production [35,37]. A significant male bias in mRNA levels was found in the head for
139 *AstA*, *AstC*, *Tk*, *NPF,* and *Dh31* (Figure 1F-1J). In contrast, mRNA levels of *AstC*, *Tk*,
140 and *NPF* in isolated intestines showed a strong female bias (Figure 1K-1M). No sex
141 bias in the expression of *AstA* or *Dh31* was observed in the gut (Figure 1N, 1O).
142 Building on the sex bias in mRNA levels, we next examined mRNA levels of
143 receptors that correspond to EE cell-expressed hormones with sex-biased expression in
144 whole-body, fat body, and head samples. Whole-body mRNA levels of the receptors for
145 AstA (*AstA-R2*), AstC (*AstC-R2*), Tk (*TkR99D*), NPF (*NPFR*), and Dh31 (*Dh31-R*) were
146 significantly higher in 5-day-old w^{1118} males compared with age-matched females
147 (Figure S1A-E). For most peptides, the male bias was due to a higher mRNA level in
148 the head and not the fat body (Figure S1A-E); however, *TkR99D* mRNA levels were
149 higher in male fat bodies with no difference in head mRNA levels (Figure S1C). Taken
150 together with our data on peptide mRNA levels, our data suggest sex differences exist
151 in both the expression of EE cell-derived hormones and in the ability of tissues to
152 respond to available peptide.
153
154 **2.2. Sex determination gene *transformer* does not regulate sex differences in EE**
155 **cell-derived peptide mRNA levels**
156 To determine the mechanism by which these differences in mRNA levels are
157 established, we tested a role for sex determination gene *transformer* (*tra*). Normally, a

158 functional Tra protein is only expressed in females, where Tra specifies most aspects of
159 female sexual development and behavior [72–75]. Indeed, ectopic Tra expression in
160 males is sufficient to specify many aspects of female sexual development and
161 physiology [7,47,48,73,76,77]. Because *tra* mRNA is detected in the gut, specifically in
162 ISC and EE cells [47,78], we asked whether broad overexpression of Tra in neurons
163 and/or EE cells contributes to the sex difference in mRNA levels of EE cell-expressed
164 hormones. We found that sex differences in mRNA levels of *AstA*, *AstC*, *Tk*, *NPF,* and
165 *Dh31* were unaffected when we used either *voila-GAL4* (Figure 2A-2J) or *elav-GAL4*
166 (Figure 2K-2T) to drive Tra expression in these cells. Tra expression in neurons
167 similarly had no effect on mRNA levels of *AstC-R2*, *TkR99D*, or *Dh31-R* in the head
168 (Figure S2A-C). Thus, sex determination *tra* does not establish sex differences in levels
169 of the mRNAs that encode either EE cell-derived hormones or their receptors.
170

171 **2.3. Gut-derived Tachykinin and Allatostatin C promote female fat storage**

172 Given that EE cell-derived AstC, NPF, and Tk regulate fat storage and phenotypes
173 associated with fat storage (e.g., starvation resistance) in single- and mixed-sex animal
174 groups [22,29,44,71], we wanted to assess whether these hormones contribute to the
175 sex difference in fat storage. We used RNAi to knock down levels of *AstC, Tk* and *NPF*
176 with GAL4 drivers targeting these specific EE populations (*AstC-GAL4, Tk-GAL4,* and
177 *NPF-GAL4*, respectively). Importantly, GAL4 activity for each driver line was restricted
178 to the gut using *R57C10-GAL80*, a validated approach to target only the gut cells that
179 produce these hormones [22,44,71]. We found gut-specific loss of *AstC* (genotype
180 *AstC-GAL4>UAS-AstC-RNAi, R57C10-GAL80*) caused a significant reduction in body

181 fat in females with no effect in males (Figure 3A). Gut-specific knockdown of *Tk*
182 (genotype *Tk-GAL4>UAS-Tk-RNAi, R57C10-GAL80*) similarly showed a trend toward a
183 female-specific decrease in fat storage (GAL4 control p=0.1109; UAS control
184 p=0.0118), with no significant effect on male body fat (Figure 3B). In contrast, gut-
185 specific loss of *NPF* (*NPF-GAL4>UAS-NPF-RNAi, R57C10-GAL80*) did not significantly
186 alter whole-body fat storage in either males or females (Figure 3C). Together, these
187 data suggest that the female-biased expression of AstC and Tk in the gut play
188 physiologically significant roles in regulating fat storage in females.
189
190 **2.4. Allatostatin C receptor and Tachykinin receptor in neurons promote fat**
191 **storage in females but not males**
192 Neurons and neuropeptide-producing cells are key cell types upon which AstC, Tk, and
193 NPF act to influence physiology [22,29,32,71,79,80]. We therefore predicted that loss of
194 *AstC-R2* and *TkR99D* in these cells would reproduce the reduced fat storage we
195 observed in females with loss of EE cell-derived AstC and Tk. To test this, we used
196 *elav-GAL4* to knock down *AstC-R2* and *TkR99D* in post-mitotic neurons and
197 neuropeptide-producing cells. In females, loss of *AstC-R2* in neurons and neuropeptide-
198 producing cells caused a significant decrease in body fat, with no effect in males (Figure
199 4A). This reproduced the body fat phenotype caused by loss of gut AstC. A similar
200 female-specific reduction in fat storage was observed with loss of *TkR99D* in neurons
201 and neuropeptide-producing cells (Figure 4B), reproducing the fat storage phenotype of
202 females with loss of gut-derived Tk. In line with the lack of body fat effect due to loss of
203 gut-derived NPF, we saw no significant change in fat storage in either males or females

204 with loss of *NPFR* in neurons and neuropeptide-producing cells (Figure 4C). Together,
205 these data suggest that AstC and Tk may promote whole-body fat storage in females
206 via effects on neurons and neuropeptide-producing cells.

207 To narrow down the neurons and neuropeptide-producing cells in which *AstC-R2*
208 and *TkR99D* act to mediate their effects on female fat storage, we used cell-type-
209 specific GAL4 drivers to overexpress RNAi transgenes directed at these genes. Given
210 that these gut-derived peptides have been shown to influence metabolic homeostasis
211 and feeding via effects on the insulin-producing cells (IPCs) and the Akh-producing cells
212 (APCs) [19,22,67], we first knocked down *AstC-R2* and *TkR99D* in these cells. We used
213 *dilp2-GAL4* to drive expression of *UAS-Astc-R2-RNAi* and *UAS-TkR99D-RNAi* in the
214 IPCs, and *Akh-GAL4* to drive expression of these transgenes in the APCs. Loss of
215 *AstC-R2* in the IPC had no significant effect on fat storage in either males or females
216 compared with sex-matched controls (Figure 4D). IPC-specific loss of *TkR99D*, on the
217 other hand, caused a significant increase in whole-body fat storage in males (Figure 4E)
218 with no change in females. In the APC, loss of neither receptor altered fat storage in
219 males or females (Figure 4F, 4G). Thus, while Tk normally restricts male body fat via
220 effects in the IPC, AstC and Tk promote female body fat via neurons other than the IPC
221 and APC.

222

223 **3. DISCUSSION**

224 EE cell-derived hormones regulate body fat in single- and mixed-sex animal groups;
225 however, it has been unclear whether the regulation and function of these peptides
226 differ between the sexes. The goal of our study was to perform a detailed comparison of

227 EE cell-derived hormones between the sexes, and to test if these hormones contribute
228 to the sex difference in fat storage. Our assessment revealed profound female-biased
229 expression of EE cell-expressed hormones within the gut. This differential expression
230 was physiologically significant, as we showed that EE cell-derived Tk and AstC promote
231 female fat storage. Taken together, our data provide additional insight into the
232 mechanism(s) by which unmated female flies achieve higher fat storage than male flies.
233 While it was not the main goal of our study, our survey of EE cell-expressed
234 hormones in *Drosophila* revealed that the sex bias in expression was not uniform across
235 tissues. In the gut, mRNA levels of *AstC*, *Tk*, and *NPF* were higher in females than in
236 males. In the brain, mRNA levels of these hormones showed a significant male bias, in
237 line with data from previous reports on Tk [56] and NPF [63]. While it remains unclear
238 whether the tissue-specific sex bias in expression is physiologically significant, peptides
239 derived from the gut and the brain have been shown to mediate distinct effects on
240 physiology and/or behavior. For example, EE cell-derived AstC regulates energy
241 homeostasis and food-seeking behaviors in adult females [22], whereas neuron-derived
242 AstC is involved in regulating locomotion in adult males [59] and the circadian regulation
243 of oogenesis in adult females [69]. Neuron-derived Tk similarly regulates locomotion
244 [64,68], food consumption [67], Dilp secretion [19], and aggression [56], whereas gut-
245 derived Tk regulates intestinal lipogenesis [71] and stem cell divisions in the midgut
246 [81–83]. Supporting a potential sex-specific role for peptides derived from different
247 anatomical sites in regulating physiology, gut-derived AstC stimulates fat breakdown
248 during starvation through the Akh pathway in mated females with no effect in males
249 [22]. Future studies are therefore needed to determine whether there are sex

250 differences in whether the effects of EE cell-derived hormones are primarily mediated
251 by local or systemic mechanisms.
252 Another important task for future studies will be to elucidate how sex differences
253 in neuropeptide expression are established. The first step in understanding these
254 mechanisms will be to determine which factors specify the sex bias in neuropeptide
255 mRNA levels. Because our data shows that sex determination gene *tra* does not
256 regulate the sex bias in neuropeptide expression in either the brain or the gut, the role
257 of other factors that influence sexual identity and sexual differentiation must be
258 assessed. One strong candidate is the steroid hormone ecdysone, as virgin females
259 have higher ecdysone titers than males [84–86]. Ecdysone plays a role in regulating
260 sexual differentiation and development [5,87,88], and contributes to male-female
261 differences in multiple aspects of intestinal physiology (e.g., intestinal stem cell
262 proliferation) [45,49] and brain development [89,90]. Another candidate is juvenile
263 hormone, which has been shown to regulate sexual maturation in *Drosophila* and other
264 insects [91–97]. While it remains unclear whether juvenile hormone titers differ between
265 virgin males and females, juvenile hormone regulates many aspects of gut physiology in
266 mated females (e.g., intestinal lipid accumulation, ISC proliferation) [53,98] and
267 influences brain development [99]. Other than hormones, it is possible that sex
268 determination gene *Sex-lethal* plays a role in regulating the sex difference in mRNA
269 levels of EE cell-derived hormones, as *tra*-independent effects of *Sex-lethal* have been
270 described in the brain [100].
271 In parallel to identifying the factor(s) responsible for establishing the sex
272 difference in EE cell-expressed hormones, it will be important to reveal the cellular basis

273 for this differential expression. For example, a sex difference in the number of EE cells
274 and neuropeptide-expressing cells in the brain could explain the differences in
275 expression. Supporting this, gut length, overall brain size, and neuron number have
276 been shown to differ between males and females [34,47,101–108]. In the gut, the
277 difference in length is at least partially due to a sex difference in the proliferation of
278 intestinal stem cells [45,47,48], which undergo asymmetric divisions and subsequent
279 differentiation to generate all gut cell types including EE cells [34,42,109–113].
280 In the brain, males and females differ in the number of neurons found within
281 many identified clusters [101,102,104–108,114], including the cells that produce NPF
282 [63] and Tk [56]. Differences in neuron number have been primarily attributed to sex-
283 specific programmed cell death [107,114–117]; however, sex differences in neuroblast
284 cell death and/or proliferation may also play a role [106,118–120].
285 Beyond the effects of cell number, sex differences in EE cell-derived hormone
286 mRNA levels may also be due to differential gene and/or protein expression of these
287 factors, which have been reported for other peptide hormones [7,18,63]. Because sex
288 differences in the activity of peptide hormone-producing cells have also been previously
289 described [7], it is clear that a detailed examination of sex differences in EE cells, and
290 more generally in neuropeptide-producing cells, is needed to gain a comprehensive
291 picture of how these cells differ between males and females. Benefits of such a detailed
292 study include gaining insight into potential mechanisms underlying sex differences in
293 other aspects of physiology and behavior. For example, EE cells regulate ISC
294 homeostasis [81,121], and EE cell-derived hormones act locally and systemically to
295 regulate appetite, food ingestion, food digestion, gut motility, and immune responses

296 [35,80,122,123]. Importantly, male-female differences in many of these phenotypes
297 have been reported [51,52,124–127].
298 Overall, our findings identify EE cell-derived hormones AstC and Tk as important
299 factors that promote higher fat storage in *Drosophila* adult virgin females. This builds on
300 a recent paper identifying a key role for IIS in promoting higher levels of fat storage in
301 unmated females but not males [18], advancing knowledge of the factors that establish
302 an optimal level of stored fat in each sex.
303

304 **4. MATERIALS AND METHODS**

305 **4.1. Fly strains**

306 The following fly strains from the Bloomington *Drosophila* Stock Center were used: w^{1118}
307 (#3605), *voila-GAL4* (#80572), *dilp2-GAL4* (IPCs) (#37516), *UAS-tra*^{F} (#4590), *UAS-*
308 *NPF-RNAi* (#27237), *UAS-TkR99D-RNAi* (#27513), *UAS-AstC-RNAi* (#25868), *UAS-*
309 *NPFR-RNAi* (#25939), *UAS-AstC-R2-RNAi* (#36888), *UAS-Tk-RNAi* (#25800), *elav-*
310 *GAL4* (#458). We obtained *R57C10-GAL80; NPF-GAL4, R57C10-GAL80; Tk-GAL4 and*
311 *R57C10-GAL80; AstC-GAL4* as kind gifts from Dr. Kim Rewitz at the University of
312 Copenhagen, and *Akh-GAL4* was a kind gift from Dr. Mike Gordon at The University of
313 British Columbia.
314

315 **4.2. Fly husbandry**

316 Fly media was prepared with the following ingredients: 20.5 g/L sucrose, 70.9 g/L D-
317 glucose, 48.5 g/L cornmeal, 45.3 g/L yeast, 4.55 g/L agar, 0.5 g/L $CaCl_2$•$2H_2O$, 0.5 g/L
318 $MgSO_4$•$7H_2O$, 11.77 mL/L acid mix (propionic acid/phosphoric acid). For all

experiments, we allowed female flies to lay eggs on grape juice agar plates for 12 hr. At 24 hr after egg laying, 50 larvae were picked into vials containing 10 mL of food and reared at 22°C. Males and females were distinguished by the presence of sex combs in the late pupal period and placed into single-sex vials to eclose. After eclosion, adult flies were maintained at a density of twenty flies per vial in single-sex groups. Unless otherwise stated, all experiments used 5- to 7-day-old unmated flies.

4.3. Adult weight

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

4.4. Whole-body triglyceride measurements

One biological replicate consisted of five flies. Flies were collected in a 1.5 ml tube and homogenized in 350 µl of 0.1% Tween (Amresco, 0777-1L) in 1X phosphate-buffered saline (PBS; Sigma-Aldrich, P5493) using 50 µl of glass beads (Sigma-Aldrich, Z250473) that were agitated at 8 m/s for 5 s (OMNI International BeadRuptor 24). Triglyceride concentration was measured using the Stanbio Triglyceride Liquid Reagent (FT7610, BD386a/d, BD386b) according to the manufacturer's instructions and as described previously [13,18] with minor modifications. Briefly, 10 µl of either homogenate or triglyceride standard (FT7610) was added to 190 µl of activated triglyceride reagent (Enzymatic Triglyceride Reagent, BD386a/d; Triglyceride Activator, Cat. No. BD386b) in a 96-well plate. After a 15 min incubation at room temperature, the

342 absorbance was read at 540 nm (Thermo Scientific – Multiskan FC Microplate
343 Photometer).
344

345 **4.5. RNA extraction, cDNA synthesis, and Quantitative real-time PCR (qPCR)**

346 One biological replicate consisted of 3-5 adult fly guts, or 10 adult fly heads, or 5 whole-
347 body adult flies. Samples were homogenized in 500 µl Trizol (Thermo Fisher Scientific;
348 15596018). Chloroform was added to Trizol to separate the mixture into aqueous and
349 organic phases, and isopropanol was added to the aqueous phase (in a fresh tube) to
350 precipitate the RNA. RNA was resuspended in either 20-25 µl (for guts and heads) or
351 200 µl (for whole-body) of molecular biology grade water (Corning, 46-000-CV). RNA
352 was stored at -80°C until use. Each experiment contained 5-10 biological replicates per
353 sex and per genotype; each experiment was repeated twice.
354 For genomic DNA elimination and cDNA synthesis, an equal amount of RNA per
355 reaction was DNase-treated and reverse transcribed according to manufacturer's
356 instructions using the QuantiTect Reverse Transcription Kit (Qiagen, 205314). Relative
357 mRNA transcript levels were quantified using qPCR as described previously [6]. Data
358 were normalized to the average fold change of *Actin5C* and *β-tubulin*. For a full primer
359 list, refer to Document S1.
360

361 **4.6. Statistical analysis**

362 Statistical analyses and data presentation were completed using GraphPad Prism 9
363 (GraphPad Software, San Diego, CA, USA). All data were tested for normality using the
364 Shapiro-Wilk test. Normally-distributed data were subjected to parametric tests as

365 appropriate, including Student's *t*-test and two-way ANOVA followed by Bonferroni post-
366 hoc test. For non-normally distributed data, we used the Mann-Whitney test. For two-
367 way ANOVA involving data that do not satisfy the normality assumption, aligned rank
368 transformation was first applied using the art() function from the ARTool R package
369 [128]. Then, ANOVA was performed on the transformed data with the base R anova()
370 function. Finally, the art.con() function from the ARTool package was used to extract the
371 main as well as the interaction effects. Default parameters were used in each step of
372 the analysis. For all statistical analyses, differences were considered significant if
373 $p<0.05$.
374

375 **CRediT authorship contribution statement**

376 **Puja Biswas:** Conceptualization, Data curation, Formal analysis, Investigation,
377 Methodology, Visualization, Writing – original draft.
378 **Elizabeth J. Rideout:** Conceptualization, Funding acquisition, Methodology,
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398 and unceded territory of the Musqueam people; a privilege for which we are grateful.

399

400 **Declaration of competing interests**

401 The authors declare no competing interests.

402

403 **Appendix A. Supplementary data**

404 Document S1. Supplemental Figures S1-S4

405

406 **Data availability**

407 Table S1. Excel file containing raw data with calculations

408 Table S2. Excel file containing statistics for all data

409

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840

841 **FIGURE LEGENDS**

842 **Figure 1 – Sex differences in expression of gut-derived peptide hormones.**

843 (A-E) mRNA levels of *AstA* (p<0.0001; Student's t-test) (A), *AstC* (p=0.0002; Mann-

844 Whitney test) (B), *Tk* (p<0.0001; Student's t-test) (C), *NPF* (p=0.0001; Student's t-test)

845 (D), *Dh31* (p=0.0002; Mann-Whitney test) (E) in whole-body were significantly higher in

846 5-day-old w^{1118} males compared to females. n=7-8 biological replicates.

847 (F-J) mRNA levels of *AstA* (p<0.0001; Student's t-test) (F), *AstC* (p=0.001; Student's t-

848 test) (G), *Tk* (p<0.0001; Student's t-test) (H), *NPF* (p=0.0015; Student's t-test) (I), *Dh31*

849 (p<0.0001; Student's t-test) (J) in heads were significantly higher in 5-day-old w^{1118}

850 males compared to females. n=8-10 biological replicates.

851 (K-O) mRNA levels of *AstC* (p=0.0002; Student's t-test) (K), *Tk* (p<0.0001; Student's t-
852 test) (L), *NPF* (p=0.0006; Mann-Whitney test) (M) in guts were significantly higher in 5-
853 day-old w^{1118} females compared to males. n=7 biological replicates.
854 (N-O) mRNA levels of *AstA* (p=0.5039; Student's t-test) (N), *Dh31* (p=0.7517; Student's
855 t-test) (O) in guts were not significantly different between 5-day-old w^{1118} females and
856 males. n=7 biological replicates.
857 All data plotted as mean ± SEM. ns indicates not significant with p>0.05; ** p<0.01, ***
858 p<0.001, **** p<0.0001.
859 See also Figure S1.
860
861 **Figure 2 – Sex determination gene *transformer* does not regulate sex differences**
862 **in EE cell-derived peptide mRNA levels.**
863 (A) mRNA levels of *AstA* in gut were not significantly different between *voila-*
864 *GAL4>UAS-tra*^{F} females and *voila-GAL4>+* and *+>UAS-tra*^{F} control females (p>0.9999
865 and p>0.9999, respectively). mRNA levels of *AstA* in gut were significantly higher in
866 *voila-GAL4>UAS-tra*^{F} males compared to *+>UAS-tra*^{F} control males, but were not
867 significantly different from *voila-GAL4>+* control males (p=0.0084 and p>0.9999,
868 respectively) (sex:genotype interaction p<0.0001). Two-way ANOVA followed by
869 Bonferroni post-hoc test; n=5 biological replicates.
870 (B) mRNA levels of *AstC* in gut were not significantly different between *voila-*
871 *GAL4>UAS-tra*^{F} females and *voila-GAL4>+* and *+>UAS-tra*^{F} control females (p=0.6814
872 and p=1.0, respectively). mRNA levels of *AstC* in gut were significantly higher in *voila-*
873 *GAL4>UAS-tra*^{F} males compared to *+>UAS-tra*^{F} control males, but were not

874 significantly different from *voila-GAL4*>+ control males (p=0.0463 and p=0.9965,
875 respectively) (sex:genotype interaction p=0.1078). Two-way ANOVA followed by Tukey
876 HSD on data processed using the aligned rank transform for non-parametric data; n=5
877 biological replicates.
878 (C) mRNA levels of *Tk* in gut were not significantly different between *voila-GAL4>UAS-*
879 *tra*^{F} females and *voila-GAL4*>+ and +>*UAS-tra*^{F} control females (p=0.2258 and
880 p>0.9999, respectively). mRNA levels of *Tk* in gut were significantly higher in *voila-*
881 *GAL4>UAS-tra*^{F} males compared to +>*UAS-tra*^{F} control males, but were significantly
882 lower compared to *voila-GAL4*>+ control males (p=0.0004 and p=0.0006, respectively)
883 (sex:genotype interaction p=0.0004). Two-way ANOVA followed by Bonferroni post-hoc
884 test; n=5 biological replicates.
885 (D) mRNA levels of *NPF* in gut were not significantly different between *voila-*
886 *GAL4>UAS-tra*^{F} females and *voila-GAL4*>+ and +>*UAS-tra*^{F} control females (p=0.1579
887 and p=0.3389, respectively). mRNA levels of *NPF* in gut were not significantly different
888 between *voila-GAL4>UAS-tra*^{F} males and *voila-GAL4*>+ and +>*UAS-tra*^{F} control males
889 (p=0.6639 and p=0.9043, respectively) (sex:genotype interaction p=0.1655). Two-way
890 ANOVA followed by Bonferroni post-hoc test; n=5 biological replicates.
891 (E) mRNA levels of *Dh31* in gut were significantly lower in *voila-GAL4>UAS-tra*^{F}
892 females compared to *voila-GAL4*>+ control females, but were not significantly different
893 from +>*UAS-tra*^{F} control females (p=0.0439 and p=0.9745, respectively). mRNA levels
894 of *Dh31* in gut were not significantly different between *voila-GAL4>UAS-tra*^{F} males and
895 *voila-GAL4*>+ and +>*UAS-tra*^{F} control males (p=0.9953 and p=0.1370, respectively)
896 (sex:genotype interaction p=0.1945). Two-way ANOVA followed by Tukey HSD on data

897 processed using the aligned rank transform for non-parametric data; n=5 biological
898 replicates.
899 (F) mRNA levels of *AstA* in head were not significantly different between *voila-*
900 *GAL4>UAS-tra*^{F} females and *voila-GAL4>+* and *+>UAS-tra*^{F} control females (p>0.9999
901 and p=0.3344, respectively). mRNA levels of *AstA* in head were not significantly
902 different between *voila-GAL4>UAS-tra*^{F} males and *voila-GAL4>+* and *+>UAS-tra*^{F}
903 control males (p>0.9999 and p>0.9999, respectively) (sex:genotype interaction
904 p=0.2471). Two-way ANOVA followed by Bonferroni post-hoc test; n=8 biological
905 replicates.
906 (G) mRNA levels of *AstC* in head were significantly lower in *voila-GAL4>UAS-tra*^{F}
907 females compared to *+>UAS-tra*^{F} control females, but were not significantly different
908 from *voila-GAL4>+* control females (p<0.0001 and p>0.9999, respectively). mRNA
909 levels of *AstC* in head were significantly lower in *voila-GAL4>UAS-tra*^{F} males compared
910 to *+>UAS-tra*^{F} control males, but were not significantly different from *voila-GAL4>+*
911 control males (p=0.0236 and p=0.6687, respectively) (sex:genotype interaction
912 p=0.0189). Two-way ANOVA followed by Bonferroni post-hoc test; n=8 biological
913 replicates.
914 (H) mRNA levels of *Tk* in head were significantly lower in *voila-GAL4>UAS-tra*^{F} females
915 compared to *+>UAS-tra*^{F} control females, but were not significantly different from *voila-*
916 *GAL4>+* control females (p=0.0044 and p>0.9999, respectively). mRNA levels of *Tk* in
917 head were significantly higher in *voila-GAL4>UAS-tra*^{F} males compared to both *voila-*
918 *GAL4>+* and *+>UAS-traF* control males (p=0.0120 and p=0.0156, respectively)

919 (sex:genotype interaction p=0.0003). Two-way ANOVA followed by Bonferroni post-hoc
920 test; n=8 biological replicates.
921 (I) mRNA levels of *NPF* in head were significantly lower in *voila-GAL4>UAS-tra^F*
922 females compared to *+>UAS-tra^F* control females, but were not significantly different
923 from *voila-GAL4>+* control females (p=0.0006 and p>0.9999, respectively). mRNA
924 levels of *NPF* in head were not significantly different between *voila-GAL4>UAS-tra^F*
925 males and *voila-GAL4>+* and *+>UAS-tra^F* control males (p=0.5030 and p=0.6158,
926 respectively) (sex:genotype interaction p=0.1408). Two-way ANOVA followed by
927 Bonferroni post-hoc test; n=8 biological replicates.
928 (J) mRNA levels of *Dh31* in head were significantly lower in *voila-GAL4>UAS-tra^F*
929 females compared to *+>UAS-tra^F* control females, but were not significantly different
930 from *voila-GAL4>+* control females (p=0.0003 and p>0.9999, respectively). mRNA
931 levels of *Dh31* in head were not significantly different between *voila-GAL4>UAS-tra^F*
932 males and *voila-GAL4>+* and *+>UAS-tra^F* control males (p>0.9999 and p>0.9999,
933 respectively) (sex:genotype interaction p=0.0403). Two-way ANOVA followed by
934 Bonferroni post-hoc test; n=8 biological replicates.
935 (K) mRNA levels of *AstA* in gut were not significantly different between *elav-*
936 *GAL4>UAS-tra^F* females and *elav-GAL4>+* and *+>UAS-tra^F* control females (p>0.9999
937 and p=0.0534, respectively). mRNA levels of *AstA* in gut were not significantly different
938 between *elav-GAL4>UAS-tra^F* males and *elav-GAL4>+* and *+>UAS-tra^F* control males
939 (p=0.5496 and p=0.1858, respectively) (sex:genotype interaction p=0.6125). Two-way
940 ANOVA followed by Bonferroni post-hoc test; n=6 biological replicates.

941 (L) mRNA levels of *AstC* in gut were not significantly different between *elav-*
942 *GAL4>UAS-tra*^{F} females and *elav-GAL4*>+ and +>*UAS-tra*^{F} control females (p=0.5948
943 and p=0.0878, respectively). mRNA levels of *AstC* in gut were not significantly different
944 between *elav-GAL4>UAS-tra*^{F} males and *elav-GAL4*>+ and +>*UAS-tra*^{F} control males
945 (p=0.1745 and p=0.1745, respectively) (sex:genotype interaction p=0.4992). Two-way
946 ANOVA followed by Tukey HSD on data processed using the aligned rank transform for
947 non-parametric data; n=6 biological replicates.
948 (M) mRNA levels of *Tk* in gut were significantly lower in *elav-GAL4>UAS-tra*^{F} females
949 compared to +>*UAS-tra*^{F} control females, but were not significantly different from *elav-*
950 *GAL4*>+ control females (p=0.0269 and p>0.9999, respectively). mRNA levels of *Tk* in
951 gut were not significantly different between *elav-GAL4>UAS-tra*^{F} males and *elav-*
952 *GAL4*>+ and +>*UAS-tra*^{F} control males (p>0.9999 and p=0.2110, respectively)
953 (sex:genotype interaction p=0.5212). Two-way ANOVA followed by Bonferroni post-hoc
954 test; n=6 biological replicates.
955 (N) mRNA levels of *NPF* in gut were not significantly different between *elav-*
956 *GAL4>UAS-tra*^{F} females and *elav-GAL4*>+ and +>*UAS-tra*^{F} control females (p>0.9999
957 and p=0.1158, respectively). mRNA levels of *NPF* in gut were not significantly different
958 between *elav-GAL4>UAS-tra*^{F} males and *elav-GAL4*>+ and +>*UAS-tra*^{F} control males
959 (p>0.9999 and p=0.6652, respectively) (sex:genotype interaction p=0.6546). Two-way
960 ANOVA followed by Bonferroni post-hoc test; n=6 biological replicates.
961 (O) mRNA levels of *Dh31* in gut were not significantly different between *elav-*
962 *GAL4>UAS-tra*^{F} females and *elav-GAL4*>+ and +>*UAS-tra*^{F} control females (p=0.5442
963 and p=0.8086, respectively). mRNA levels of *Dh31* in gut were not significantly different

964 between *elav-GAL4>UAS-tra^F* males and *elav-GAL4>+* and *+>UAS-tra^F* control males
965 (p=0.1650 and p=0.5258, respectively) (sex:genotype interaction p=0.9566). Two-way
966 ANOVA followed by Tukey HSD on data processed using the aligned rank transform for
967 non-parametric data; n=6 biological replicates.
968 (P) mRNA levels of *AstA* in head were not significantly different between *elav-*
969 *GAL4>UAS-tra^F* females and *elav-GAL4>+* and *+>UAS-tra^F* control females (p=0.9843
970 and p=0.7086, respectively). mRNA levels of *AstA* in head were not significantly
971 different between *elav-GAL4>UAS-tra^F* males and *elav-GAL4>+* and *+>UAS-tra^F* control
972 males (p=0.0628 and p=0.9936, respectively) (sex:genotype interaction p=0.0171).
973 Two-way ANOVA followed by Tukey HSD on data processed using the aligned rank
974 transform for non-parametric data; n=5-6 biological replicates.
975 (Q) mRNA levels of *AstC* in head were not significantly different between *elav-*
976 *GAL4>UAS-tra^F* females and *elav-GAL4>+* and *+>UAS-tra^F* control females (p=0.1253
977 and p=0.8540, respectively). mRNA levels of *AstC* in head were significantly lower in
978 *elav-GAL4>UAS-tra^F* males compared to *+>UAS-tra^F* control males, but were not
979 significantly different from *elav-GAL4>+* control males (p=0.0188 and p=0.9086,
980 respectively) (sex:genotype interaction p=0.0198). Two-way ANOVA followed by Tukey
981 HSD on data processed using the aligned rank transform for non-parametric data; n=5-6
982 biological replicates.
983 (R) mRNA levels of *Tk* in head were not significantly different between *elav-GAL4>UAS-*
984 *tra^F* females and *elav-GAL4>+* and *+>UAS-tra^F* control females (p=0.6051 and
985 p=0.9999, respectively). mRNA levels of *Tk* in head were not significantly different
986 between *elav-GAL4>UAS-tra^F* males and *elav-GAL4>+* and *+>UAS-tra^F* control males

987 (p=0.3600 and p=0.2760, respectively) (sex:genotype interaction p=0.2324). Two-way
988 ANOVA followed by Tukey HSD on data processed using the aligned rank transform for
989 non-parametric data; n=5-6 biological replicates.
990 (S) mRNA levels of *NPF* in head were significantly lower in *elav-GAL4>UAS-tra*^{F}
991 females compared to both *elav-GAL4>+* and *+>UAS-tra*^{F} control females (p=0.0347 and
992 p=0.0273, respectively). mRNA levels of *NPF* in head were not significantly different
993 between *elav-GAL4>UAS-tra*^{F} males and *elav-GAL4>+* and *+>UAS-tra*^{F} control males
994 (p=0.0656 and p=0.6253, respectively) (sex:genotype interaction p=0.6872). Two-way
995 ANOVA followed by Tukey HSD on data processed using the aligned rank transform for
996 non-parametric data; n=5-6 biological replicates.
997 (T) mRNA levels of *Dh31* in head were not significantly different between *elav-*
998 *GAL4>UAS-tra*^{F} females and *elav-GAL4>+* and *+>UAS-tra*^{F} control females (p>0.9999
999 and p>0.9999, respectively). mRNA levels of *Dh31* in head were not significantly
1000 different between *elav-GAL4>UAS-tra*^{F} males and *elav-GAL4>+* and *+>UAS-tra*^{F} control
1001 males (p=0.2918 and p=0.5990, respectively) (sex:genotype interaction p=0.4360).
1002 Two-way ANOVA followed by Bonferroni post-hoc test; n=5-6 biological replicates.
1003 All data plotted as mean ± SEM. ns indicates not significant with p>0.05; * p<0.05, **
1004 p<0.01, *** p<0.001, **** p<0.0001.
1005 See also Figure S2.
1006
1007 **Figure 3 – Gut-derived Tachykinin and Allatostatin C promote female fat storage.**
1008 (A) Whole-body triglyceride levels were significantly lower in *AstC-GAL4>UAS-AstC-*
1009 *RNAi,R57C10-GAL80* females compared to *AstC-GAL4>+,R57C10-GAL80 and*

1010 *+>UAS-AstC-RNAi* control females (p<0.0001 and p<0.0001, respectively). Whole-body
1011 triglyceride levels were not significantly different between *AstC-GAL4>UAS-AstC-*
1012 *RNAi*,*R57C10-GAL80* males and *AstC-GAL4>+,R57C10-GAL80* and *+>UAS-AstC-*
1013 *RNAi* control males (p=0.4527 and p=0.1056, respectively) (sex:genotype interaction
1014 p<0.0001). Two-way ANOVA followed by Bonferroni post-hoc test; n=8 biological
1015 replicates.
1016 (B) Whole-body triglyceride levels were significantly lower in *Tk-GAL4>UAS-Tk-*
1017 *RNAi*,*R57C10-GAL80* females compared to *+>UAS-Tk-RNAi* control females
1018 (p=0.0118) but were not significantly different from *Tk-GAL4>+,R57C10-GAL80* control
1019 females (p=0.1109). Whole-body triglyceride levels were significantly higher in *Tk-*
1020 *GAL4>UAS-Tk-RNAi*,*R57C10-GAL80* males compared to *Tk-GAL4>+,R57C10-GAL80*
1021 control males (p<0.0001) but were not significantly different from *+>UAS-Tk-RNAi*
1022 control males (p=0.5704) (sex:genotype interaction p<0.0001). Two-way ANOVA
1023 followed by Bonferroni post-hoc test; n=8 biological replicates.
1024 (C) Whole-body triglyceride levels were not significantly different between *NPF-*
1025 *GAL4>UAS-NPF-RNAi,R57C10-GAL80* females and *NPF-GAL4>+,R57C10-GAL80* and
1026 *+>UAS-NPF-RNAi* control females (p>0.9999 and p>0.9999, respectively). Whole-body
1027 triglyceride levels were not significantly different between *NPF-GAL4>UAS-NPF-*
1028 *RNAi*,*R57C10-GAL80* males and *NPF-GAL4>+,R57C10-GAL80* and *+>UAS-NPF-RNAi*
1029 control males (p>0.9999 and p=0.4134, respectively) (sex:genotype interaction
1030 p=0.2890). Two-way ANOVA followed by Bonferroni post-hoc test; n=8 biological
1031 replicates.

1032 All data plotted as mean ± SEM. ns indicates not significant with $p>0.05$; ** $p<0.01$, ****
1033 $p<0.0001$.
1034
1035 **Figure 4 – Allatostatin C receptor and Tachykinin receptor in neurons promote fat**
1036 **storage in females but not males.**
1037 (A) Whole-body triglyceride levels were significantly lower in *elav-GAL4>UAS-AstC-R2-*
1038 *RNAi* females compared to *elav-GAL4>+* and *+>UAS-AstC-R2-RNAi* control females
1039 ($p=0.0001$ and $p=0.0013$, respectively). Whole-body triglyceride levels were not
1040 significantly different between *elav-GAL4>UAS-AstC-R2-RNAi* males and *elav-GAL4>+*
1041 and *+>UAS-AstC-R2-RNAi* control males ($p=0.0564$ and $p>0.9999$, respectively)
1042 (sex:genotype interaction $p=0.0631$). Two-way ANOVA followed by Bonferroni post-hoc
1043 test; n=8 biological replicates.
1044 (B) Whole-body triglyceride levels were significantly lower in *elav-GAL4>UAS-TkR99D-*
1045 *RNAi* females compared to *elav-GAL4>+* and *+>UAS-TkR99D-RNAi* control females
1046 ($p<0.0001$ and $p<0.0001$, respectively). Whole-body triglyceride levels were not
1047 significantly different between *elav-GAL4>UAS-TkR99D-RNAi* males and *elav-GAL4>+*
1048 and *+>UAS-TkR99D-RNAi* control males ($p>0.9999$ and $p>0.9999$, respectively)
1049 (sex:genotype interaction $p<0.0001$). Two-way ANOVA followed by Bonferroni post-hoc
1050 test; n=8 biological replicates.
1051 (C) Whole-body triglyceride levels were significantly lower in *elav-GAL4>UAS-NPFR-*
1052 *RNAi* females compared to *elav-GAL4>+* control females ($p<0.0001$) but were not
1053 significantly different from *+>UAS-NPFR-RNAi* control females ($p>0.9999$). Whole-body
1054 triglyceride levels were significantly lower in *elav-GAL4>UAS-NPFR-RNAi* males

1055 compared to *elav-GAL4*>+ control males (p<0.0001) but were not significantly different
1056 from +>*UAS-NPFR-RNAi* control males (p>0.9999) (sex:genotype interaction
1057 p=0.2470). Two-way ANOVA followed by Bonferroni post-hoc test; n=8 biological
1058 replicates.
1059 (D) Whole-body triglyceride levels were significantly lower in *dilp2-GAL4>UAS-AstC-R2-*
1060 *RNAi* females compared to *dilp2-GAL4*>+ control females (p<0.0001) but were not
1061 significantly different from +>*UAS-AstC-R2-RNAi* control females (p>0.9999). Whole-
1062 body triglyceride levels were significantly higher in *dilp2-GAL4>UAS-AstC-R2-RNAi*
1063 males compared to *dilp2-GAL4*>+ control males (p=0.0156) but were not significantly
1064 different from +>*UAS-AstC-R2-RNAi* control males (p=0.3419) (sex:genotype interaction
1065 p<0.0001). Two-way ANOVA followed by Bonferroni post-hoc test; n=8 biological
1066 replicates.
1067 (E) Whole-body triglyceride levels were significantly lower in *dilp2-GAL4>UAS-TkR99D-*
1068 *RNAi* females compared to *dilp2-GAL4*>+ control females (p=0.0321) but were not
1069 significantly different from +>*UAS-TkR99D-RNAi* control females (p=0.0724). Whole-
1070 body triglyceride levels were significantly higher in *dilp2-GAL4>UAS-TkR99D-RNAi*
1071 males compared to *dilp2-GAL4*>+ and +>*UAS-TkR99D-RNAi* control males (p<0.0001
1072 and p=0.0003, respectively) (sex:genotype interaction p<0.0001). Two-way ANOVA
1073 followed by Bonferroni post-hoc test; n=8 biological replicates.
1074 (F) Whole-body triglyceride levels were not significantly different in *Akh-GAL4>UAS-*
1075 *AstC-R2-RNAi* females compared to *Akh-GAL4*>+ control females (p=0.3817) but were
1076 significantly lower than +>*UAS-AstC-R2-RNAi* control females (p=0.0181). Whole-body
1077 triglyceride levels were not significantly different in *Akh-GAL4>UAS-AstC-R2-RNAi*

1078 males compared to *Akh-GAL4*>+ and +>*UAS-AstC-R2-RNAi* control males (p=0.1229
1079 and p>0.9999, respectively) (sex:genotype interaction p=0.0241). Two-way ANOVA
1080 followed by Bonferroni post-hoc test; n=8 biological replicates.
1081 (G) Whole-body triglyceride levels were not significantly different in *Akh-GAL4>UAS-*
1082 *TkR99D-RNAi* females compared to *Akh-GAL4*>+ and +>*UAS-TkR99D-RNAi* control
1083 females (p=0.4601 and p>0.9999, respectively). Whole-body triglyceride levels were not
1084 significantly different in *Akh-GAL4>UAS-TkR99D-RNAi* males compared to *Akh-*
1085 *GAL4*>+ and +>*UAS-TkR99D-RNAi* control males (p>0.9999 and p=0.8744,
1086 respectively) (sex:genotype interaction p=0.0595). Two-way ANOVA followed by
1087 Bonferroni post-hoc test; n=8 biological replicates.
1088 All data plotted as mean ± SEM. ns indicates not significant with p>0.05; * p<0.05, **
1089 p<0.01, *** p<0.001, **** p<0.0001.

Figure 1

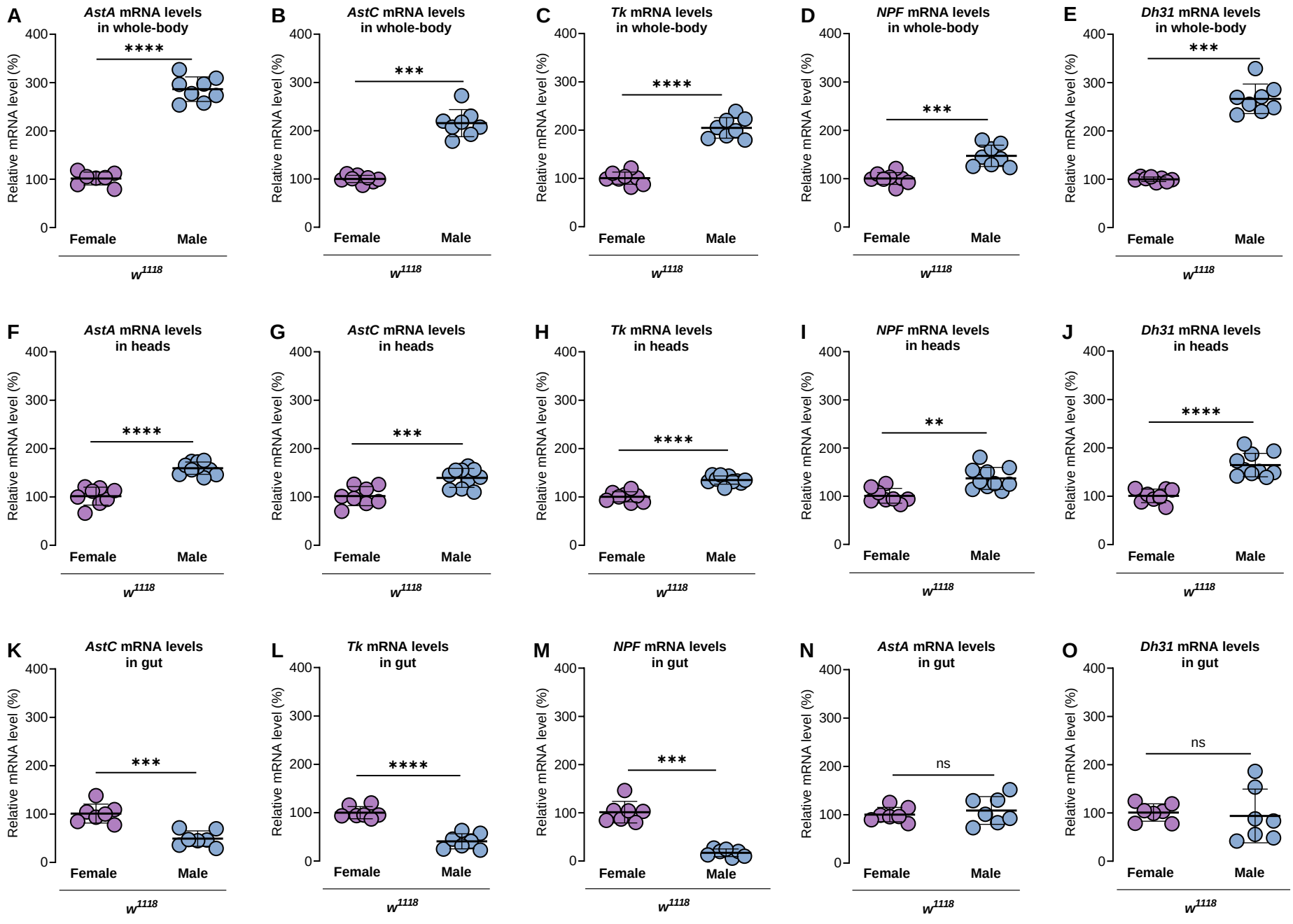

A
AstA mRNA levels in whole-body
****
B
AstC mRNA levels in whole-body
***
C
Tk mRNA levels in whole-body
****
D
NPF mRNA levels in whole-body
***
E
Dh31 mRNA levels in whole-body
***
F
AstA mRNA levels in heads
****
G
AstC mRNA levels in heads
***
H
Tk mRNA levels in heads
****
I
NPF mRNA levels in heads
**
J
Dh31 mRNA levels in heads
****
K
AstC mRNA levels in gut
***
L
Tk mRNA levels in gut
****
M
NPF mRNA levels in gut
***
N
AstA mRNA levels in gut
ns
O
Dh31 mRNA levels in gut
ns
Relative mRNA level (%)
0
100
200
300
400
Female
Male
w1118

Figure 2

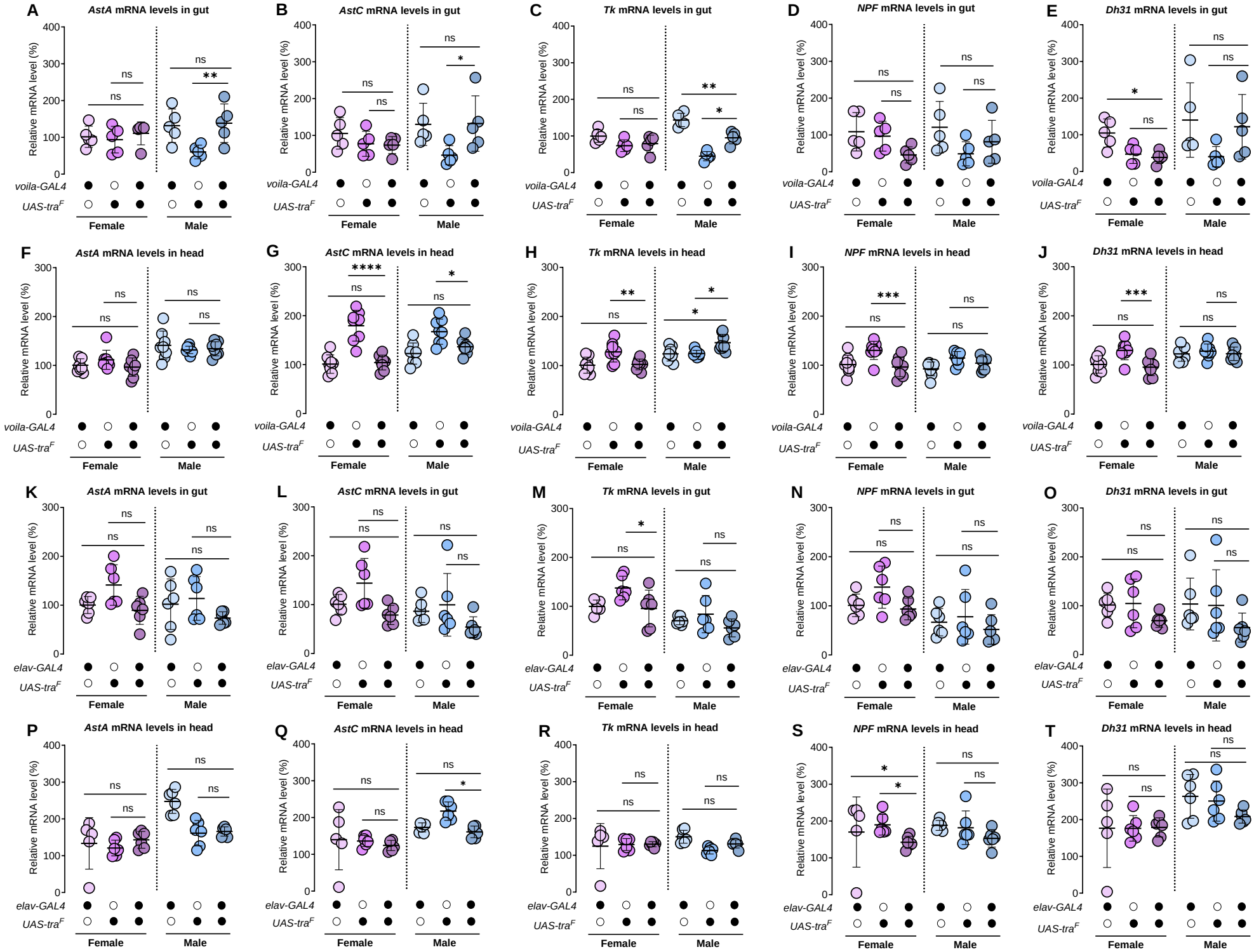

A
AstA mRNA levels in gut
B
AstC mRNA levels in gut
C
Tk mRNA levels in gut
D
NPF mRNA levels in gut
E
Dh31 mRNA levels in gut
F
AstA mRNA levels in head
G
AstC mRNA levels in head
H
Tk mRNA levels in head
I
NPF mRNA levels in head
J
Dh31 mRNA levels in head
K
AstA mRNA levels in gut
L
AstC mRNA levels in gut
M
Tk mRNA levels in gut
N
NPF mRNA levels in gut
O
Dh31 mRNA levels in gut
P
AstA mRNA levels in head
Q
AstC mRNA levels in head
R
Tk mRNA levels in head
S
NPF mRNA levels in head
T
Dh31 mRNA levels in head
Relative mRNA level (%)
voila-GAL4
elav-GAL4
UAS-traF
Female
Male
ns
*
**
***
****

Figure 3

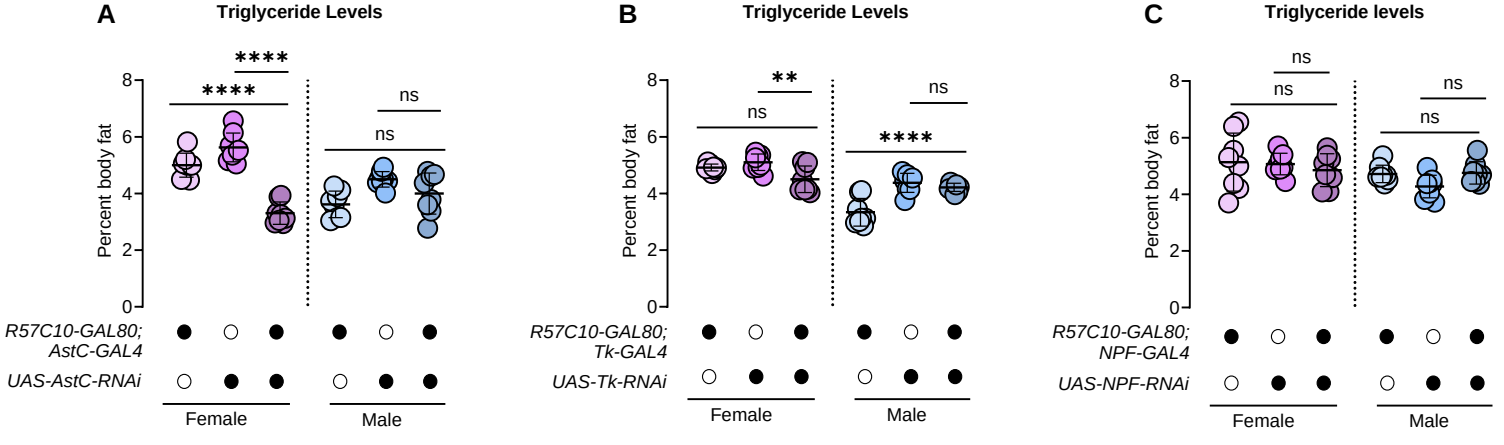
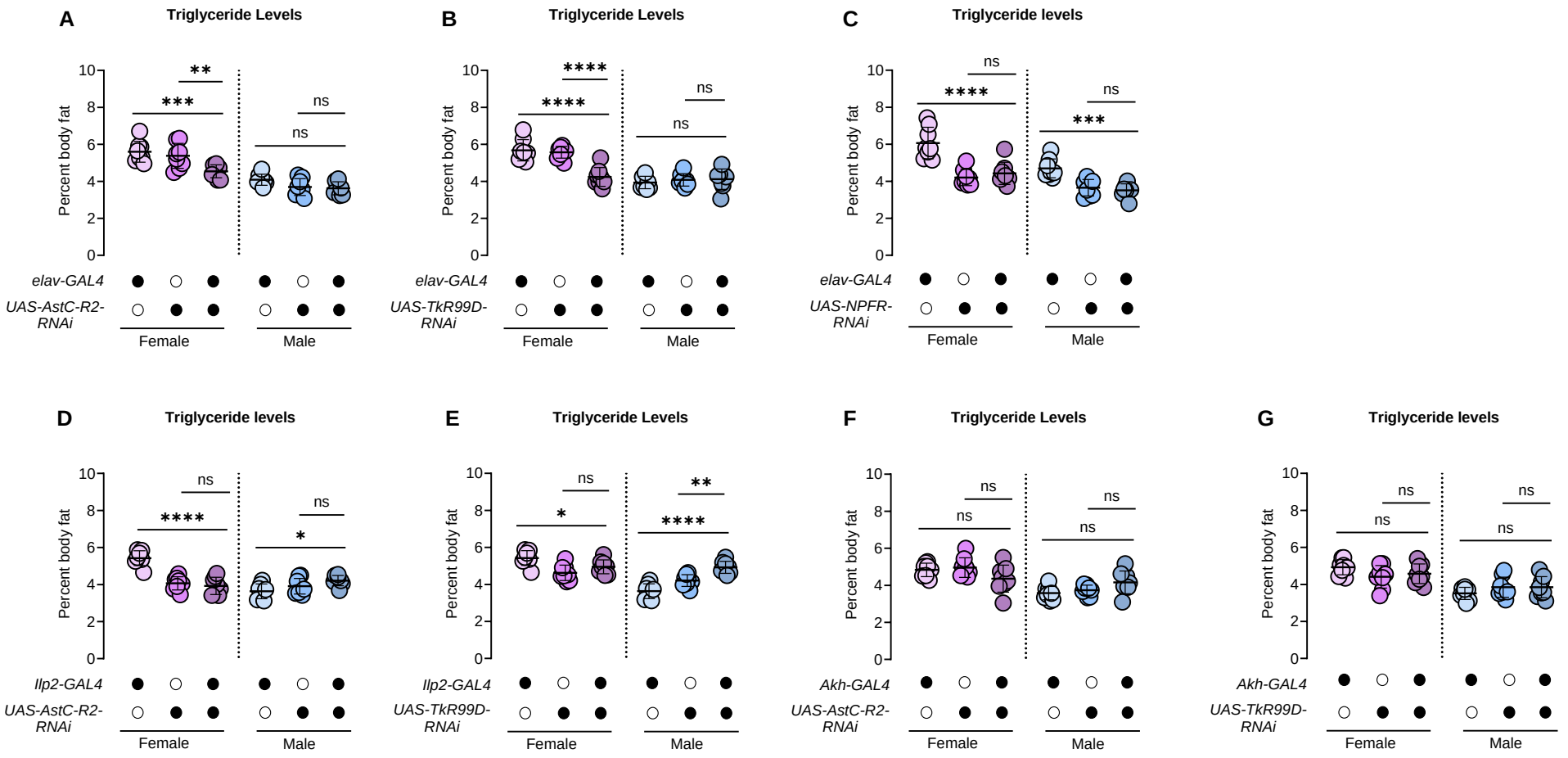

A
Triglyceride Levels
****
****
ns
ns
Percent body fat
8
6
4
2
0
R57C10-GAL80; AstC-GAL4
UAS-AstC-RNAi
Female
Male
B
Triglyceride Levels
**
ns
ns
****
Percent body fat
R57C10-GAL80; Tk-GAL4
UAS-Tk-RNAi
Female
Male
C
Triglyceride levels
ns
ns
ns
ns
Percent body fat
R57C10-GAL80; NPF-GAL4
UAS-NPF-RNAi
Female
Male

Figure 4


A
Triglyceride Levels
Percent body fat
elav-GAL4
UAS-AstC-R2-RNAi
Female
Male
B
Triglyceride Levels
Percent body fat
elav-GAL4
UAS-TkR99D-RNAi
Female
Male
C
Triglyceride levels
Percent body fat
elav-GAL4
UAS-NPFR-RNAi
Female
Male
D
Triglyceride levels
Percent body fat
Ilp2-GAL4
UAS-AstC-R2-RNAi
Female
Male
E
Triglyceride Levels
Percent body fat
Ilp2-GAL4
UAS-TkR99D-RNAi
Female
Male
F
Triglyceride Levels
Percent body fat
Akh-GAL4
UAS-AstC-R2-RNAi
Female
Male
G
Triglyceride levels
Percent body fat
Akh-GAL4
UAS-TkR99D-RNAi
Female
Male