Lipid metabolism dysfunction following symbiont elimination is linked to altered Kennedy pathway homeostasis

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Abstract

Lipid metabolism is critical for insect reproduction, especially for species that invest heavily into early developmental stages of their offspring. The role of symbiotic bacteria during this process is unknown but likely essential, especially in the case of obligate microbes that fulfill key biological functions in the host. Using a combined lipidomics, functional genomics and biochemical strategy, we examined the role of lipid metabolism in the interaction between the viviparous tsetse fly (*Glossina morsitans morsitans*) and its obligate endosymbiotic bacteria (*Wigglesworthia glossinidia*) during tsetse pregnancy. We observed increased *CTP:phosphocholine cytidylyltransferase* (*cct1*) expression during pregnancy. This gene codes for the enzyme that functions as the rate limiting step in phosphatidylcholine biosynthesis in the Kennedy pathway which is critical for stored lipid metabolism and progeny development. Experimental removal of *Wigglesworthia* impaired lipid metabolism via disruption of the Kennedy pathway, yielding obese mothers whose developing progeny ultimately starve. Functional validation via experimental *cct1* suppression revealed a phenotype similar to females lacking obligate *Wigglesworthia* symbionts. These results indicate that, in *Glossina*, symbiont-derived factors, likely B vitamins, are critical for proper function of both lipid biosynthesis and lipolysis. Loss of the symbiosis has a dramatic impact on *Glossina* fecundity, and may be broadly applicable to other insect systems, particularly to species that require symbiotic partners to maximize lipolysis and reproductive output.

Keywords: B vitamin, reproduction, *Wigglesworthia*, tsetse fly, phospholipids

Introduction

Reproduction represents an evolutionarily imperative and metabolically taxing physiological process for all animals (Flatt, 2011; Perrin and Sibly, 1993). This may be especially true for insects, which maintain a high reproductive rate, even though progeny quality declines throughout adulthood (Barreaux et al., 2022; Michalkova et al., 2014a). One such insect is the tsetse fly, *Glossina spp*., which reproduces via a process called ‘adenotrophic viviparity’ (gland fed, live birth (Benoit et al., 2015)). Female tsetse ovulate one egg per gonotrophic cycle and following fertilization and embryogenesis, larvigenesis proceeds within the maternal uterus. *In utero,* larvae are nourished exclusively by milk secretions produced by uniquely adapted maternal accessory glands, called the milk glands. Thus, female tsetse flies supply all the nutrients necessary to support embryonic and larval development of their progeny. Tsetse milk consists of 20-30 mg of carbohydrates, lipids and proteins in an aqueous base (Benoit et al., 2015, 2014; Cmelik et al., 1969; Tobe, 1978). The rapid incorporation of nutrients into milk products during lactation reduces total maternal lipid and protein content by nearly 50% and 25%, respectively (Attardo et al., 2012). Following birth, the milk glands rapidly involute and lipid reserves accumulate to support a subsequent offspring (Attardo et al., 2012; Baumann et al., 2013; Benoit et al., 2018). Tsetse mothers endure this metabolically intense K-selected reproductive strategy continuously through their adulthood, ultimately birthing up to 8-12 larvae per lifetime (Barreaux et al., 2022; Michalkova et al., 2014a).

Tsetse flies have evolved to feed exclusively on vertebrate blood. While blood is nutrient-rich, it lacks in sufficient quantity several of the vitamins and cofactors required

70 to support the metabolically intense process of milk production. To overcome this,
71 tsetse flies have developed intimate, long-term associations with symbiotic bacteria that
72 supplement nutrients missing from host blood. One of these bacteria is the maternally
73 transmitted obligate endosymbiont *Wigglesworthia glossinidia*, which has associated
74 exclusively with tsetse species for 50-80 million years (Chen et al., 1999). As a result,
75 *Wigglesworthia* has experienced massive genome erosion (Akman et al., 2002; Hall et
76 al., 2019). However, the bacterium retains genes required to produce a variety of B
77 vitamins that are largely absent from vertebrate blood (Akman et al., 2002; Bing et al.,
78 2017). Metabolic processing of proline, which circulates in tsetse hemolymph and
79 replaces carbohydrates as the flies' prominent energy source, is fueled by B vitamins
80 generated by *Wigglesworthia* (Michalkova et al., 2014b). Accordingly, antibiotic-
81 mediated clearance of *Wigglesworthia* results in a dramatic reduction in multiple B
82 vitamin-associated compounds that serve as essential cofactors in fundamental
83 metabolic pathways, including the pentose phosphate pathway, nucleotide biosynthesis,
84 methionine/cysteine metabolism, and amino acid and lipid metabolism. These
85 deficiencies yield adult female flies incapable of milk production which consequently
86 results in abortion of early instar larvae (Benoit et al., 2017; Bing et al., 2017; Nogge,
87 1976). However, the mechanistic basis for this phenotype remains unclear.

88 Here we examined the interplay among obligate symbionts, lipid metabolism, and
89 reproductive output of tsetse flies. Maternal transfer of nutrients to developing offspring
90 is an energetically demanding process for all insects. However, tsetse flies, similar to
91 other viviparous systems, invest even more into their progeny by providing resources for
92 embryonic and larval development. This study demonstrates that critical symbiont-

93 derived factors promote phospholipid synthesis, facilitating rapid lipolysis of stored lipid
94 reserves in order to nourish the developing intrauterine progeny. Other insects that rely
95 on symbiont-derived B vitamins, including bed bugs and lice (Benoit et al., 2016; Boyd
96 et al., 2017; Hosokawa et al., 2010; Kirkness et al., 2010), are likely to experience
97 impaired lipolysis and reduced fecundity in the absences of their symbiotic partner(s),
98 as the molecular mechanisms underlying this process are not unique to *Glossina*
99 (Thomas et al., 2020). Furthermore, similarities in live birth across animal systems
100 indicate that micronutrient deprivation is likely to have significant impacts on metabolic
101 processes with similar negative consequences that extend from invertebrates to
102 vertebrates (Fouks et al., 2022).
103

104 **Results**

105 Aposymbiotic flies are obese and fail to reproduce

106 Antibiotic-treated, endosymbiont-free (aposymbiotic) tsetse flies failed to reproduce
107 successfully, as previously shown (Benoit et al., 2017; Michalkova et al., 2014b; Nogge,
108 1976). When early instar larvae from aposymbiotic mothers were removed before
109 abortion and compared to control larvae, their lipid levels were 25-30% lower, indicating
110 substantially reduced lipid transfer from mother to larvae in the aposymbiotic state (Fig.
111 1). Aposymbiotic females show higher total lipid levels, with an average of 5.66 ± 0.44
112 lipids per mg dry mass relative to 4.85 ± 0.34 in symbiotic females. This phenotype
113 was associated with increased lipid droplet (LD) size in the fat body (FB), suggesting
114 defect(s) in lipolysis. Additionally, Nile blue staining of FB tissue revealed a reduction in

115 charged phospholipids relative to neutral storage lipid species, including tri- and di-
116 glycerides, in aposymbiotic flies relative to control flies (Fig. 1). These initial studies
117 suggest a major dysfunction in lipid metabolism following symbiont clearance.
118
119 Lipidomics reveals massive shifts in metabolic lipid processing
120 To understand the biochemical landscape facilitating the accumulation of
121 triacylglycerols in endosymbiont-free flies, we generated aposymbiotic females via
122 tetracycline application, and analyzed pooled FB and milk gland (MG) tissues,
123 comparing aposymbiotic and untreated (control), age-matched females. Analysis of the
124 differential biochemical composition revealed substantial alterations in the composition
125 of multiple biochemical categories in aposymbiotic flies, including B vitamins,
126 creatine/creatinine, amino acids, diacylglycerols (DAG), phospholipids, sphingolipids,
127 lysolipids and polyunsaturated fatty acids (Suppl. Figs. 1-6). These changes yield an
128 overall composition difference in the lipid profiles between control and aposymbiotic flies
129 (Suppl. Fig. 7, ANOSIM, R = 0.879, P = 1.03E-06).
130 Many of the observed changes are via either direct or indirect effects of
131 dysregulation of phospholipid (PL) biosynthesis via the Kennedy pathway (Fig. 2).
132 Notably, aposymbiotic flies were deficient in key precursors to PL synthesis including
133 choline, phosphocholine, and the majority of the detected DAGs, and
134 phosphatidylcholines (PCs). Many DAG variants are present in adult female tsetse, and
135 they differ in acyl chain lengths and desaturation state. The most abundant DAGs in
136 control flies contain 16/18 carbon unsaturated or mono-unsaturated side chains (Fig. 2
137 and Suppl. Fig. 2), while aposymbiotic flies show a broad reduction in abundance of

138 these DAGs. Among phosphatidylcholines, aposymbiotic flies generally lack moieties
139 containing 16/18 carbon unsaturated or mono-unsaturated side chains, while those
140 containing 18/20 carbon polyunsaturated fatty acids are abundant (Suppl. Fig. 1). This
141 may reflect the observed overabundance of polyunsaturated free fatty acids in
142 aposymbiotic flies (Suppl. Fig. 4). The substantial deficiencies in the precursor
143 molecules required for phosphatidylcholine production suggest that *Wigglesworthia*-
144 derived factors either directly or indirectly facilitate the production of phospholipid
145 precursors.

146 Aposymbiotic flies also demonstrate a profound deficiency in creatine and
147 creatinine (Suppl. Fig. 6). Creatine/creatinine biosynthesis and the urea cycle are
148 dependent on folate, which is provided to tsetse flies by *Wigglesworthia* (Snyder and
149 Rio, 2015). Components of the urea cycle are also reduced in abundance in
150 aposymbiotic flies. The urea cycle is also dependent on folate for proper function
151 (Brosnan and Brosnan, 2010; Fatterpaker et al., 1951). Creatine/creatinine are key
152 regulators of lipid homeostasis for adrenergic and diet-induced thermogenesis in brown
153 and beige adipose tissue in vertebrates. Induced deficiencies in in creatine/creatinine in
154 mice results in diet induced obesity, which can be rescued by dietary creatine
155 supplementation (Kazak et al., 2017). The mechanism behind how these compounds
156 regulate lipolysis remains to be elucidated but may also be involved in maintenance of
157 lipid homeostasis throughout the reproductive cycle.
158
159

160 Choline-phosphate cytidylyltransferase 1 represents a critical enzyme during tsetse fly
161 reproductive cycles

162 Prior RNA-seq analyses of gene expression during tsetse pregnancy and milk
163 production revealed a suite of highly-expressed milk protein-encoding genes (Attardo et
164 al., 2019; Benoit et al., 2014; International Glossina Genome Initiative, 2014). Notably,
165 transcripts for the enzyme choline-phosphate cytidylyltransferase 1 (*cct1*) were
166 upregulated in a similar manner to milk proteins in pregnant flies (Fig. 3). This enzyme
167 participates in phosphatidylcholine synthesis. Phosphatidyl cholines are critical for
168 regulation of LD size and formation to facilitate lipolysis during energy-intensive
169 processes (Krahmer et al., 2011). The expression of *cct1* increases during milk gland
170 involution following parturition (Fig. 3). Tissue-specific expression analysis confirmed
171 *cct1* transcripts were abundant in the FB and MG. In support of this finding, *in situ*
172 hybridization revealed *cct1* expression in both of these tissues (Fig. 3C). These data
173 suggest that *cct1* is critical for lipid metabolism during tsetse pregnancy, likely
174 functioning in the FB and MG, to break down lipids critical as energy for milk production,
175 or necessary to produce milk LDs.

176

177 Suppression of cct1 phenocopies the aposymbiotic state

178 To confirm if impaired *cct1* function and altered phospholipid metabolism perturbs lipid
179 metabolism during viviparity, we suppressed *cct1* expression using RNA interference
180 (RNAi) (Fig. 4). Similar to aposymbiotic flies, *cct1* knockdown flies produce few progeny
181 (Fig. 4). We noted a massive decrease in levels of phospholipids and PC in developing
182 larvae, indicating that *cct1* is necessary to ensure sufficient lipid transfer to the

183 developing larva. This decrease likely accounts for the 40-45% reduction in total lipids
184 measured from larvae deposited from *cct1* knockdown flies (0.20 ± 0.03 mg lipid/ mg
185 dry mass for *cct1* RNAi vs 0.29 ± 0.04 for control). In aposymbiotic females, *cct1*
186 expression increases substantially, perhaps as a compensatory response to reduced
187 phospholipid levels, which are required for lipid transfer during pregnancy.
188 Tetracycline treatment impairs mitochondrial function (Moullan et al., 2015),
189 which could represent an off-target outcome that significantly impacts tsetse lipid
190 metabolism. As such, we treated flies with glyphosate, which specifically inhibits
191 *Wigglesworthia* folate production (Rio et al., 2019). This yielded flies that presented
192 obesity measures similar to their aposymbiotic counterparts (Fig. 4G). Lastly,
193 aposymbiotic flies were fed on blood with a cocktail of B vitamins and yeast extract,
194 which reduced the lipid levels within the flies, providing a direct link between B vitamins
195 and obesity levels (Fig. 4H). This finding confirmed that *Wigglesworthia*-derived B
196 vitamins acts as an essential factor for tsetse lipid homeostasis.
197

198 **Discussion**

199 This study defines a novel feature of host-symbiont dynamics in tsetse showing
200 dysfunctional PL metabolism as a resultant phenotype of symbiont elimination (Fig. 5).
201 Tsetse fly pregnancy requires a rapid transfer of substantial nutritional resources from
202 mother to offspring. A key mechanism in this process is the production of PC by the
203 Kennedy biosynthesis pathway. The *cct1* gene encodes a rate-limiting enzyme in this
204 pathway that prepares the choline headgroup for attachment to cytidyl triphosphate to

205 generate CDP-choline. CDP-choline is then combined with diacylglycerol to produce the
206 final phosphatidyl choline product. The observed increase in *cct1* transcripts during
207 pregnancy suggests that this enzyme/pathway is critical to provision of PC for LD
208 production/function for lipolysis in the FB and for incorporation into products secreted
209 from the MG (Benoit et al., 2015; International Glossina Genome Initiative, 2014).
210 Production of PC is required to coat the outer surface of LDs produced by the
211 endoplasmic reticulum, and is thus critical for lipid metabolism (Olzmann and Carvalho,
212 2019). The amphipathic nature of PC prevents aberrant LD fusion and provides the
213 biochemical conditions to recruit LD-associated proteins required for lipolysis (Pol et al.,
214 2014).

215 In mammals, disruption of appropriate PC to PE ratios in LDs causes formation
216 of large droplets with reduced surface area and altered surface protein composition.
217 The end result is abnormal fat retention within a cell or tissue/organ (steatosis;
218 (Listenberger et al., 2018). In tsetse flies, symbiont clearance produces obesity, similar
219 to that observed in mammals where individuals see increased lipid levels in conjunction
220 with symbiont dysfunction. Flies initially fed blood spiked with tetracycline are cleared of
221 their *Wigglesworthia*, with any lingering bacterial-derived factors clearing through
222 subsequent feedings. Importantly, multiple feeding events following tetracycline
223 treatment over the course of two weeks reduces the direct impact of this treatment,
224 indicating that the phenotypes are associated with the loss of the symbiont. Symbiont
225 absence results in PC deficiency that inhibits metabolism, mobilization and integration
226 of stored lipids during lactation. Reproduction is terminated due to lipid retention in the
227 FB and inefficient lipid transfer to the intrauterine larva. This is likely due to the

228 combined effect of the mothers inability to process FB lipids due to dysfunction in lipid
229 droplet formation in the FB and MG as *cct1* is abundantly expressed in both locations.
230 The biochemical observations associated with this phenotype suggest at least
231 two points of failure in phosphatidylcholine biosynthesis via the Kennedy (CDP-choline)
232 pathway. The first limiting factor appears to be deficiencies in choline and choline
233 phosphate levels, which form the phosphatidylcholine headgroup. The production of
234 choline is dependent on methylation reactions driven by enzymes that utilize S-adenosyl
235 methionine (SAM) as a cofactor. Prior work in aposymbiotic *Glossina* demonstrates a
236 severe deficiency of SAM in aposymbiotic flies (Bing et al., 2017). Production of SAM is
237 dependent on the cysteine-methionine pathway, which is dependent on the B vitamins
238 pyridoxal and folate as enzymatic cofactors (Loenen, 2006; Nguyen et al., 2001).
239 Methylation reactions require SAM as a cofactor for the production of choline and
240 conversion of phosphatidylethanolamine (PE) to PC (Gibellini and Smith, 2010; Mazen
241 Noureddin et al., 2015; Snyder and Rio, 2015). In accordance with the observations in
242 mammalian systems, the mechanistic disruption occurring in the aposymbiotic flies is
243 most likely due to the deficiency in symbiont-derived B vitamins.
244 *Wigglesworthia* produces an array of B vitamins (B1, B2, B3, B5, B6, B7 and B9)
245 (Akman et al., 2002; Bing et al., 2017; Rio et al., 2019; Snyder and Rio, 2015).
246 Deficiencies in B6, folate and, consequently, SAM have a negative impact on
247 phospholipid biosynthesis in mammalian systems. Issues related to the production of
248 phosphatidylcholine have been linked to folate restriction (Abratte et al., 2008). The
249 aposymbiotic tsetse phenotype bears resemblance to the pathology observed in non-
250 alcoholic fatty liver disease (NAFLD) in mammals as PC deficiency is a key dysfunction

251 (Bianca M. Arendt et al., 2013; Jacobs et al., 2013; Ville Männistö et al., 2019). Rats
252 exhibiting a NAFLD phenotype have been rescued by dietary supplementation with
253 phosphatidylcholine (Kitagawa et al., 2015). The phenotype observed in aposymbiotic
254 flies (this study and in prior studies (Bing et al., 2017; Snyder and Rio, 2015)) is
255 phenotypically equivalent to that observed when *cct1* is suppressed. Folate deficiency is
256 likely a major underlying factor for the phenotype of dysfunctional metabolism of lipids
257 we observe in flies lacking symbionts. Induction of folate deficiency in tsetse through
258 treatment with glyphosate impacts vector competency and reproduction (Rio et al.,
259 2019; Snyder and Rio, 2015), highlighting that folate provision by *Wigglesworthia*
260 represents a critical lynchpin in tsetse metabolism. While female tsetse are able to
261 survive B vitamin deficiency, the metabolic deficiencies prevent them from performing
262 the mass mobilization of lipids from FB stores to the MG required for milk production
263 during pregnancy. Our supplementation of B vitamins to aposymbiotic flies reduces
264 obesity and has been previously shown to recover fecundity (Michalkova et al., 2014b),
265 providing a direct link between B vitamins provided by *Wigglesworthia* and obesity in
266 *Glossina*. This is not surprising as *cct* is overexpressed in aposymbiotic lines, indicating
267 once specific B vitamins are available lipolysis can occur more rapidly to support
268 reproduction.

269 Another disruption in phosphatidylcholine biosynthesis appears to derive from
270 DAG deficiency. The lack of DAGs results in inadequate substrate availability for fusion
271 with cytidine 5'-diphosphocholine by DAG cholinephosphotransferase. DAGs can be
272 synthesized *de novo* during the production of triglycerides or they can be produced
273 during lipolysis of triglycerides upon removal of a fatty acid side chain by a lipase.

274 Triglycerides are overabundant in aposymbiotic flies, suggesting that lipolysis of the
275 existing triglycerides is compromised under these conditions. In insects, the lipase
276 responsible for this activity is called *brummer lipase* (*Drosophila*, CG5295), and is
277 orthologous to the Adipose triglyceride lipase (ATGL) in mammals (Alexander Mildner et
278 al., 2005; Grönke et al., 2007). Brummer and ATGL are both regulated by the
279 phosphorylation state of other lipid droplet bound proteins, called perilipins (Sztalryd
280 and Brasaemle, 2017). Perilipins sequester a co-activator of ATGL in their non-
281 phosphorylated state called CGI-58, which inhibits lipolysis (Itabe et al., 2017). Recent
282 work demonstrates that LDs lacking proper phosphatidylcholine levels show increased
283 levels of associated perilipins and a steatosis (lipid accumulation) phenotype
284 (Listenberger et al., 2018). The phosphatidylcholine deficiencies in aposymbiotic
285 *Glossina* could be functioning via the same paradigm, resulting in perilipin accumulation
286 and inhibition of *brummer*-mediated lipolysis resulting in DAG deficiency and TAG
287 accumulation.

288 The symbiosis between tsetse flies and *Wigglesworthia* is essential for this
289 complex system and both are dependent on one another for their survival. These
290 studies demonstrate that lipid metabolism, a foundational process in *Glossina* biology, is
291 impaired in the absence of their obligate symbionts. This is likely a model for other
292 symbiotic systems, particularly those compensating for dietary nutritional deficiencies
293 such as blood feeding invertebrates that are supplied with B vitamins from bacterial
294 symbionts. This includes ticks (Duron et al., 2018), bed bugs (Nikoh et al., 2014), and
295 lice (Boyd et al., 2017), which are all epidemiologically important blood-feeding disease
296 vectors and require obligate symbionts to provide B vitamins. Beyond blood-feeding

297 arthropods, specific bacteria associated with plant feeding insects also provide B
298 vitamins (Salem et al., 2014), suggesting that lipid metabolism and B vitamin
299 dependency issues likely occur in many systems. Lastly, B vitamins can be provided to
300 insect species that have transient bacterial symbionts, such as in mosquitoes (Wang et
301 al., 2021), suggesting that the interdependence of B vitamins and lipid metabolism may
302 be a widespread phenomenon among arthropod species. As a key example,
303 mosquitoes display altered lipid metabolism related to microbiome elimination and B
304 vitamin deficiency (Didion et al., 2021; Romoli et al., 2021; Valzania et al., 2018; Wang
305 et al., 2021). Specifically, the mosquitoes fail to accumulate lipids at critical time points,
306 such as early in development and when lipids are accumulated for dormancy (Didion et
307 al., 2021; Romoli et al., 2021; Valzania et al., 2018; Wang et al., 2021), indicating a
308 critical role for B vitamins, lipid metabolism, and symbiont presence for many biological
309 processes, including the critical contribution to reproduction we observed in this study.
310 Along with the role among arthropod systems, genomic studies have identified
311 significant functional convergence among live-bearing systems in invertebrate and
312 vertebrate lineages (Fouks et al., 2022). This includes aspects that range from
313 metabolism to immune function (Fouks et al., 2022). In the vertebrate systems, fetal
314 development requires essential fatty acids and long chain polyunsaturated fatty acids
315 (Herrera, 2002; Knopp et al., 1998; Ryckman et al., 2015). As in the insect system, the
316 accumulation of lipids early in vertebrate pregnancy is a critical feature that builds up
317 stores providing lipids to the developing progeny (Herrera, 2002). As pregnancy
318 progresses, the mother transitions from lipogenesis to lipolysis to increase the lipid
319 supply to the fetus (Herrera et al., 1994, 1988). An imbalance in lipid levels provided to

320 the neonates, either through malnutrition of the mother or impaired transfers, can yield
321 many negative consequences ranging from delayed growth/development to death
322 (Furness et al., 2012; Herrera, 2002). Similar to this study, B vitamin levels have been
323 documented to alter lipid metabolism in mammalian systems with most focus on human
324 and murine systems (Castaño-Moreno et al., 2020; da Silva et al., 2020; Herrera, 2002).
325 Low B vitamin levels during pregnancy have been directly associated with a reduction in
326 circulating lipid levels, increased obesity in the mother, and a reduction in health of the
327 neonate (Adaikalakoteswari et al., 2017; Castaño-Moreno et al., 2020; McNeil et al.,
328 2009), which is functionally similar to the obesity and reduced larval growth we
329 observed in tsetse flies lacking B vitamins produced by *Wigglesworthia*. As observed in
330 this study for tsetse flies, the induction of obesity acts through impaired PC synthesis
331 that requires specific B vitamins, which is also critical for vertebrate lipid metabolism (da
332 Silva et al., 2014). Thus, a distinct commonality of viviparity among animals is the
333 requirements of specific B vitamins for lipid metabolism to meet the energetic demand
334 of internal progeny development. This expands on genomic similarities (Fouks et al.
335 2022) to other physiological processes that are similar between invertebrate and
336 vertebrate viviparity.
337

338 **Methods**

339 Flies

340 *Glossina morsitans morsitans* flies were maintained on defibrinated bovine blood
341 (Lampire Biologicals, Pipersville, PA, USA) at 25°C and 50-60% RH using an artificial
342 membrane feeding system (Moloo, 1971) at the insectaries at Yale School of Public

343 Health and the Slovak Academy of Sciences. Females were mated 3-5 days after
344 emergence. Flies were collected according to established developmental markers
345 based on oocyte, embryo and larva presence (Attardo et al., 2006; Strickler-Dinglasan
346 et al., 2006; Yang et al., 2010). Aposymbiotic tsetse larvae were derived from females
347 fed a diet supplemented with tetracycline (20 µg per ml of blood) to clear their
348 indigenous microbiota and yeast extract (1%) to provide vitamins in the absence of
349 obligate *Wigglesworthia* (Pais et al., 2008). A subset of the progeny were allowed to
350 feed of blood supplemented with B vitamins and yeast extract according to Michalkova
351 et al. (2014b), which has been shown to partially alleviate B vitamin deficiencies
352 following *Wigglesworthia* elimination.
353
354
355 Milk extraction
356 Milk products were extracted from the guts of developing 3rd instar larvae based on our
357 previous studies (Benoit et al., 2014, 2012). Briefly, actively-feeding individuals were
358 removed from the uterus and their digestive tracts were dissected. A pulled glass
359 capillary needle was utilized to pierce into the digestive tract and the contents were
360 removed using reverse pressure. Each sample was stored at -80ºC until utilization in the
361 experiments.
362
363 Lipidomics
364 Tissues (milk glands and fat body) were dissected from aged-matched WT flies that
365 received normal blood meals for five weeks (control - symbiotic) and aposymbiotic

366 females and prepared according to our previously established protocol (Bing et al.,
367 2017). Briefly, tissues were added 100 µl of ice cold 1X phosphate buffered saline
368 (PBS), homogenized with a pestle and stored at -80° C. For both aposymbiotic and
369 control flies, samples consisted of four replicates of 20 individuals each. Samples were
370 shipped to Metabolon (Morrisville, NC, USA) and analysis was performed using the
371 proprietary Metabolon DiscoveryHD4 global lipidomics platform. Each sample was
372 screened for the presence and quantity of lipids by mass spectrometry using a panel
373 specific lipids. Relative differences between samples were determined by comparison of
374 mean metabolite abundance across the biological replicates. Significance of the
375 difference between symbiotic and aposymbiotic samples was determined by Welch's
376 two-sample t-test: p-value cut off of (<0.05). False discovery rate (FDR) was tested for
377 by q-value with a cut off of (<0.1). Non-Metric Multidimensional Scaling (NMDS) plots
378 were created using the metaMDS function from the R package vegan (Dixon, 2003;
379 Oksanen, 2007) to determine differences in the general lipid composition between
380 control and aposymbiotic individuals. Analysis of similarities (ANOSIM, using the
381 anosim from the R package vegan) tests was performed to test for significance in the
382 general differences.
383
384 <u>RNA extraction and qPCR</u>
385 RNA was extracted from flies with TRizol reagent (Invitrogen, Carlsbad, CA, USA) using
386 the manufacturer's recommended protocol. RNA was treated with TURBO DNA Free kit
387 (Ambion, Austin, TX, USA). RNA was precipitated with alcohol to remove residual salt,

388 purified with a RNeasy kit (Qiagen, Maryland, USA) and stored at -70ºC until use. cDNA
389 was prepared using the Superscript III reverse transcriptase (Invitrogen).
390 Transcript abundance was determined utilizing quantitative PCR (qPCR) on a Bio-Rad
391 CFX detection system (Hercules). Primer sequences used were those from Table S1.
392 Results were analyzed with CFX manager software version 3.1 (Bio-Rad). Ct values for
393 genes of interest were standardized by Ct values for the control gene (tubulin) and
394 relative to the average value for the control treatment or newly emerged flies, yielding a
395 relative fold change in gene expression.
396
397 RNA interference of *cct1*
398 Suppression of *cct1* was performed utilizing double stranded RNA (dsRNA) according to
399 our previous studies on tsetse gene suppression (Benoit et al., 2017, 2012). The T7
400 promoter sequence was added to the 5′ end of the primer sequences and PCR
401 amplification conditions are described (Table S1). The PCR products were purified
402 using QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into pGEM T-
403 Easy vector (Promega, Madison, WI) and verified by sequencing (Keck DNA
404 sequencing facility, Yale University). dsRNAs were synthesized using the MEGAscript
405 RNAi Kit (Ambion, Austin, TX), purified using a RNeasy Mini Kit (Qiagen, Valencia, CA)
406 and siRNAs were generated by using the Block-iT Dicer RNAi kit (Invitrogen, Carlsbad,
407 CA). The siRNA concentration was adjusted to 600–800 ng/µl in PBS and each fly was
408 injected with 1.5 µl siRNA using a pulled glass capillary needle into their thorax.
409 Concentration of siRNAs were kept at 600–800 ng/µl within the 1.5 µl injected.
410 Knockdown efficiency was determined 5d after injection by qPCR and normalized to

411 *tubulin* levels. Fecundity following *cct1* was assessed according to previously
412 established protocols (Benoit et al., 2014, 2012).
413
414 Glyphosate treatment
415 Groups of *G. morsitans* flies were maintained on a diet containing 100 μM glyphosate
416 100 μM glyphosate combined with 500 nM folic acid [N-(phosphonomethyl)glycine;
417 Sigma-Aldrich, St. Louis, MO, USA] or (Sigma-Aldrich). This treatment has been
418 previously documented to impact folate synthesis (Rio et al., 2019; Snyder and Rio,
419 2015). Following six bloodmeals, the flies were examined to determine if lipid and
420 phosphotydilcholine levels were increased or decreased. These treatments were
421 conducted to eliminate off-target effects of tetracycline supplementation validate
422 Wigglesworthia's role in tsetse lipid metabolism provide a secondary validation of
423 impaired lipid metabolism without treatment with antibiotics.
424
425 Total lipid assay
426 Total lipids present within biological samples were determined with a standard vanillin
427 assay (Attardo et al., 2012; Rosendale et al., 2019; Van Handel, 1985). Whole flies and
428 individual tissues were dried at 0% RH at 60ºC and weighed to determine the dry mass.
429 The flies were homogenized in 2 ml of chloroform:methanol (2:1). The supernatant was
430 removed and placed into a glass tube and the solvent was evaporated at 90ºC. The
431 dried lipids were treated with 0.4 ml of concentrated sulfuric acid at 90ºC for 10 min. The
432 acid/lipid mixture (40 μl) was combined with 4 ml vanillin reagent. Samples were

433 measured spectrophotometrically at 525 nm and the total lipid content was calculated
434 against a lipid standard.
435
436 *In situ* hybridization for *cct1*
437 Milk gland tubules and fat body were removed from tsetse fly females at six-seven days
438 after the previous birth. The combined milk gland/fat body was placed into Carnoy's
439 fixative for a five-six day fixation period (Attardo et al., 2008; Michalkova et al., 2014a).
440 Antisense/sense digoxigenin-labeled RNA probes for *cct1* were generated using the
441 MAXIscript T7 transcription kit following manufacturer's protocol (Ambion, Austin, TX,
442 USA) using a primer set with a T7 primer (Table S1). Antibody solutions were made
443 using α-Digoxigenin-rhodamine, Fab fragments (Roche Applied Science, Penzberg,
444 Germany) for FISH probe detection (1:200 dilution) and rabbit α-GmmMGP (1:2500)
445 antibodies. Alexa Fluor 488 goat α-rabbit IgG (Invitrogen) at a dilution of 1:500 was
446 added as a secondary antibody for immunohistochemistry (Attardo et al., 2008;
447 Michalkova et al., 2014a). Slides were mounted in VECTASHIELD Mounting Medium
448 with DAPI (Vector laboratories Inc. Burlingame, CA, USA). Samples were observed
449 using a Zeiss Axioskop2 microscope (Zeiss, Thornwood, NY, USA) equipped with a
450 fluorescent filter and viewed and imaged at 400x magnification. Images were captured
451 using an Infinity1 USB 2.0 camera and software (Lumenera Corporation, Ottawa,
452 Ontario, Canada) and merged in Adobe Photoshop.
453
454 Phosphatidylcholine assay

455 Phosphatidylcholine levels were assessed through the use of a colorimetric assay
456 (Caymen Chemicals, Ann Arbor, MI, USA). Extracted milk from the guts of 3rd instar
457 larvae (10 µl) was placed within the sample well of a 96 well plate. The colorimetric
458 reaction was initiated by adding 100 µl of the reaction mixture. Following mixing for a
459 few seconds, the plate was incubated at 37°C for 60 min and concentration determined
460 by comparison to a phosphatidylcholine standard.
461
462 Nile Blue staining
463 Lipid composition of fat body cells from control and aposymbiotic *Glossina* was
464 analyzed microscopically using the lipophilic Nile Blue stain. Nile Blue is a differential
465 stain, which results in charged phospholipids (fatty acids, chromolipids and
466 phospholipids) being stained blue and neutral lipids (triglycerides, cholesterol esters,
467 steroids) being stained red/pink. The method used here is a modified version of the
468 protocol described by Canete and colleagues (Canete et al., 1983). Fat body tissues
469 were dissected from flies reared as described in the Lipidomics section above.
470 Dissected tissues were immediately transferred to ice cold 4% paraformaldehyde in
471 PBS and allowed to fix overnight at 4°C. Fixed tissues were then rinsed in PBS followed
472 by immersion in 0.1 mg/ml aqueous Nile Blue sulfate (Sigma-Aldrich) for 30 min. After
473 staining, tissues were rinsed three times with PBS for 1 min per rinse to remove excess
474 stain. Tissues were then mounted in glycerol on single depression concave microscope
475 slides. Stained fat bodies were visualized using a Zeiss Axio Vert.A1 scope and images
476 captured using the Zeiss Zen imaging software. Color differences were quantified by

477 measuring the average amount of red and blue color in five 100 pixel squares within the
478 center of each lipid droplet with the use of Adobe Photoshop.
479
480 Statistical analyses
481 Lipidomics data were analyzed using the Metaboanalyst software package
482 (https://www.metaboanalyst.ca/) (Pang et al., 2021). Metabolite abundance values
483 represent peak area counts measured from compound peaks. Samples were
484 normalized across samples using the sum of raw area counts for each sample. Missing
485 values were imputed by replacement with minimum detected values for each
486 metabolite. Data were rescaled by median centering to a value of 1 for each sample.
487 Significant differences between control and aposymbiotic samples were measured by t-
488 test followed by p-value correction using false discovery rate (FDR)/Benjamini and
489 Hochberg analysis. Differences in relative abundance were considered significant below
490 an FDR corrected P-value of 0.05. Differences in other biological attributes were
491 assessed through the use of t-test or ANOVA.
492
493
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500 **Figure legends**

501 **Figure 1:** Reproduction and lipid metabolism is dysfunctional in aposymbiotic flies.
502 Proportion of females giving birth (A), N = 4, and lipid content in the surviving larvae (B)
503 are significantly reduced in aposymbiotic flies (t-test, P-value 0.007 and 0.0003,
504 respectively). N = 8-10. C-H) Nile Blue staining reveals significant differences in the
505 neutral and charged lipid composition in aposymbiotic and control flies. I) Volume of the
506 fat body is increased significantly in aposymbiotic flies (t-test, P-value 0.008). N = 12-14.
507 J) Image quantification reveals significantly higher charged lipids in the control
508 compared to the aposymbiotic flies (t-test, P-value 0.002). N = 12-14.

509

510 **Figure 2:** Lipidomics reveals a significant shift in lipid moetities following symbiont
511 removal. Compounds showing a significantly different fold change in their abundance in
512 control (yellow) and aposymbiotic (green) flies are shown. Red denotes increase in
513 compounds of the Kennedy pathway in aposymbiotic flies, while blue indicates lipids
514 showing higher fold change in control individuals. Specifically, there is a major
515 dysfunction in the Kennedy pathway for phosphatidylcholine synthesis. N = 5, *
516 indicates significance based on a t-test.

517

518 **Figure 3:** Increased expression of *choline-phosphate cytidylyltransferase 1* (*cct1*) is
519 associated with tsetse fly pregnancy. A) Previous RNA-seq studies (Attardo et al., 2019;
520 Benoit et al., 2014) reveal that *cct1* is expressed in pregnant/lactating flies. B) qPCR
521 indicated that the expression pattern for *cct1* is correlated with the pregnancy cycle,
522 similar to a milk protein critical to feed the developing larvae (*milk gland protein 1*,

523 *mgp1*). N = 4. C) Expression of *cct1* is localized in both the milk glands and fat body,
524 suggesting critical roles in lipid breakdown and milk production. N = 4, *, denotes
525 significance based on a Kal's proportion test or t-test.
526
527 **Figure 4:** Suppression of *cct1* produces aposymbiotic phenotypes of altered lipid
528 metabolism during pregnancy. A) RNA interference reduces the expression of *cct1*. N =
529 5, *, denotes significance based on a t-test. *Mgp1, milk gland protein 1.* B-C) Total
530 progeny production and progeny generated per day are reduced following suppression
531 of *cct1*. N = 3 groups of 10. Different letters denote significance based on an ANOVA.
532 *Acid smase1*, *asmase1*. D-E) Milk phospholipids and phosphatidylcholine are reduced
533 when *cct1* is reduced. N = 12. *, denotes significance based on a t-test. F) Aposymbiotic
534 flies show increased expression of *cct1* as a potential compensatory mechanism to
535 allow for lipolysis during pregnancy. N = 10. *, denotes significance based on a t-test. G)
536 Glyphosate treatment increased lipid levels within flies. N = 8. *, denotes significance
537 based on a t-test. H) Blood supplementation with B vitamins or yeast extract reduced
538 obesity in flies. N = 10. *, denotes significance based on ANOVA.
539
540 **Figure 5:** Summary of the dynamics between symbiont loss and reproduction during
541 tsetse fly pregnancy in relation to altered lipolysis. The percentage annotations in the
542 panel depicting the Kennedy Pathway represent the relative amount of the specific lipid
543 metabolite in the aposymbiotic tsetse fly relative to the untreated, wild-type control
544 which would be equivalent to 100%.
545

546 **Supplemental Figure 1:** Absolute abundance of detected fat body/milk gland
547 phospholipids and associated compounds in symbiotic and aposymbiotic *G. morsitans.*
548 Abundance values represent normalized area under compound peaks. Significance
549 determined by Welch's Two-Sample t-test followed by FDR adjustment of resulting p-
550 values.
551
552 **Supplemental Figure 2:** Absolute abundance of detected fat body/milk gland
553 diacylglycerols in symbiotic and aposymbiotic *G. morsitans.* Abundance values
554 represent normalized area under compound peaks. Significance determined by Welch's
555 Two-Sample t-test followed by FDR adjustment of resulting p-values.
556
557 **Supplemental Figure 3:** Absolute abundance of detected fat body/milk gland
558 sphingolipids and associated compounds in symbiotic and aposymbiotic *G. morsitans.*
559 Abundance values represent normalized area under compound peaks. Significance
560 determined by Welch's Two-Sample t-test followed by FDR adjustment of resulting p-
561 values.
562
563 **Supplemental Figure 4:** Absolute abundance of detected fat body/milk gland
564 polyunsaturated fatty acids (PUFAs) in symbiotic and aposymbiotic *G. morsitans.*
565 Abundance values represent normalized area under compound peaks. Significance
566 determined by Welch's Two-Sample t-test followed by FDR adjustment of resulting p-
567 values.
568

569 **Supplemental Figure 5:** Absolute abundance of detected fat body/milk gland lysolipids
570 in symbiotic and aposymbiotic *G. morsitans.* Abundance values represent normalized
571 area under compound peaks. Significance determined by Welch's Two-Sample t-test
572 followed by FDR adjustment of resulting p-values.
573
574 **Supplemental Figure 6:** Absolute abundance of detected fat body/milk gland amino
575 acid and vitamin B associated metabolic pathways undergoing significant changes in
576 abundance between in symbiotic and aposymbiotic *G. morsitans.* Abundance values
577 represent normalized area under compound peaks. Significance determined by Welch's
578 Two-Sample t-test followed by FDR adjustment of resulting p-values.
579
580 **Supplemental Figure 7:** NMDS analysis comparing the lipid profile differences
581 between aposymbiotic and control flies. Significant variation between aposymbiotic and
582 control samples (ANOSIM, R = 0.879, P = 1.03E-06).
583
584 **Table S1:** Primers used for qPCR, generation of dsRNA, and *in situ* hybridization.
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866

Figure 1

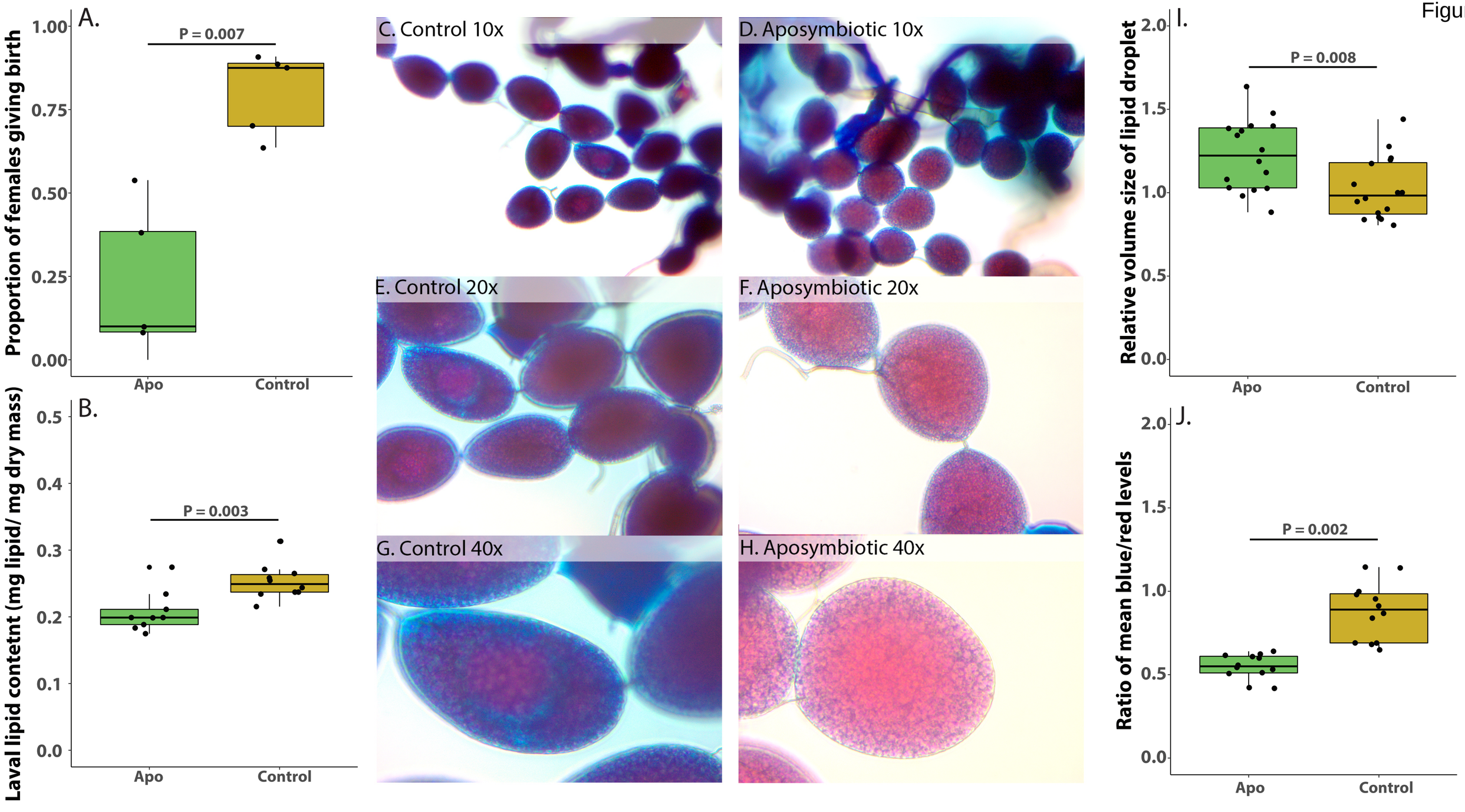
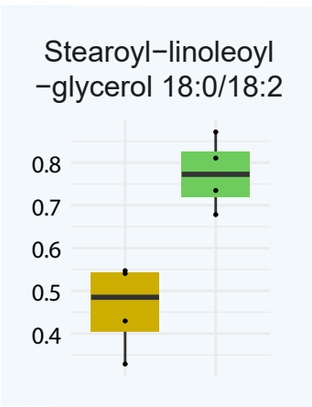

A.
P = 0.007
Proportion of females giving birth
1.00
0.75
0.50
0.25
0.00
Apo
Control
B.
P = 0.003
Laval lipid contetnt (mg lipid/ mg dry mass)
0.5
0.4
0.3
0.2
0.1
0.0
Apo
Control
C. Control 10x
D. Aposymbiotic 10x
E. Control 20x
F. Aposymbiotic 20x
G. Control 40x
H. Aposymbiotic 40x
I.
P = 0.008
Relative volume size of lipid droplet
2.0
1.5
1.0
0.5
0.0
Apo
Control
J.
P = 0.002
Ratio of mean blue/red levels
2.0
1.5
1.0
0.5
0.0
Apo
Control

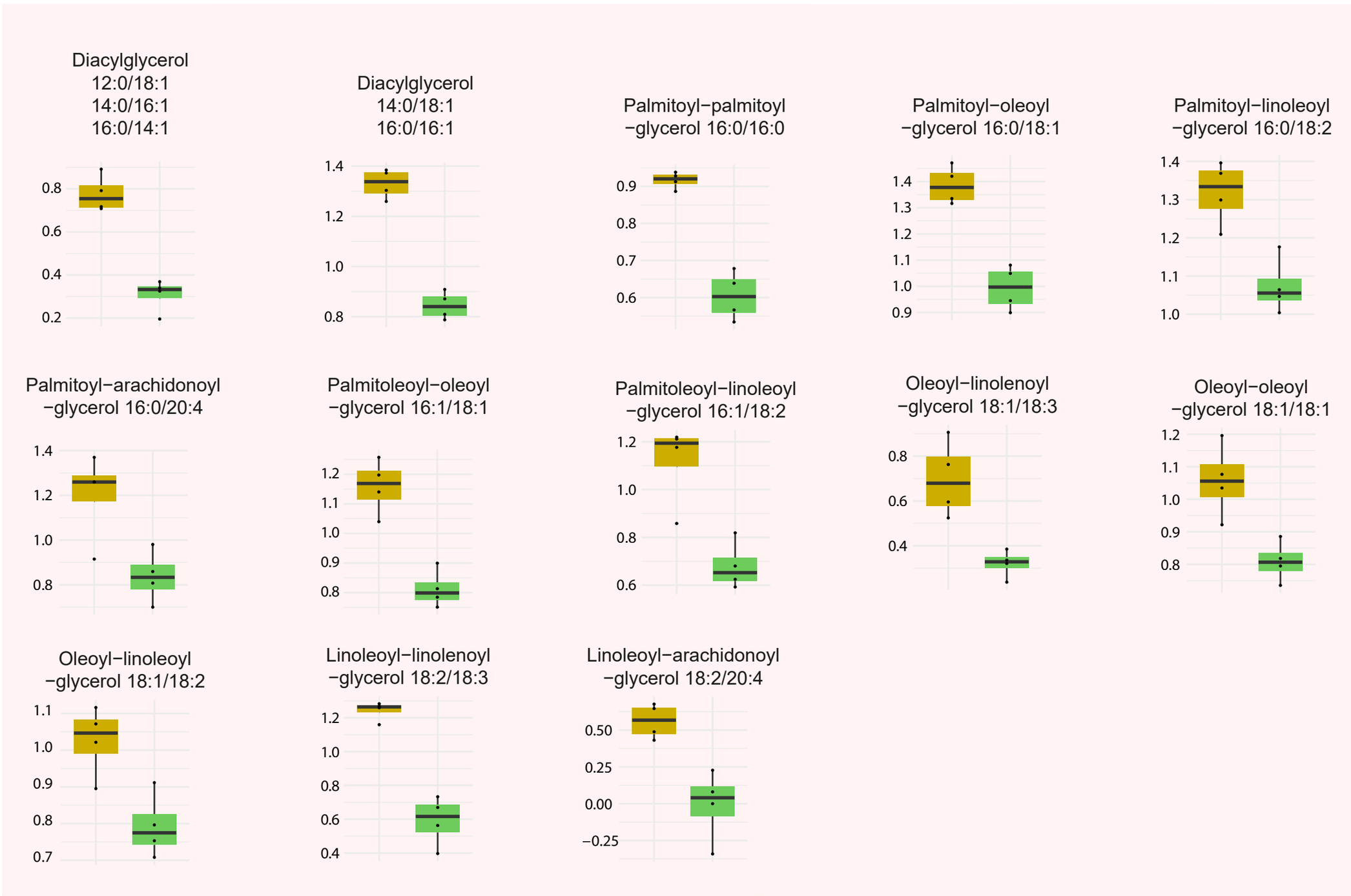
Figure 2

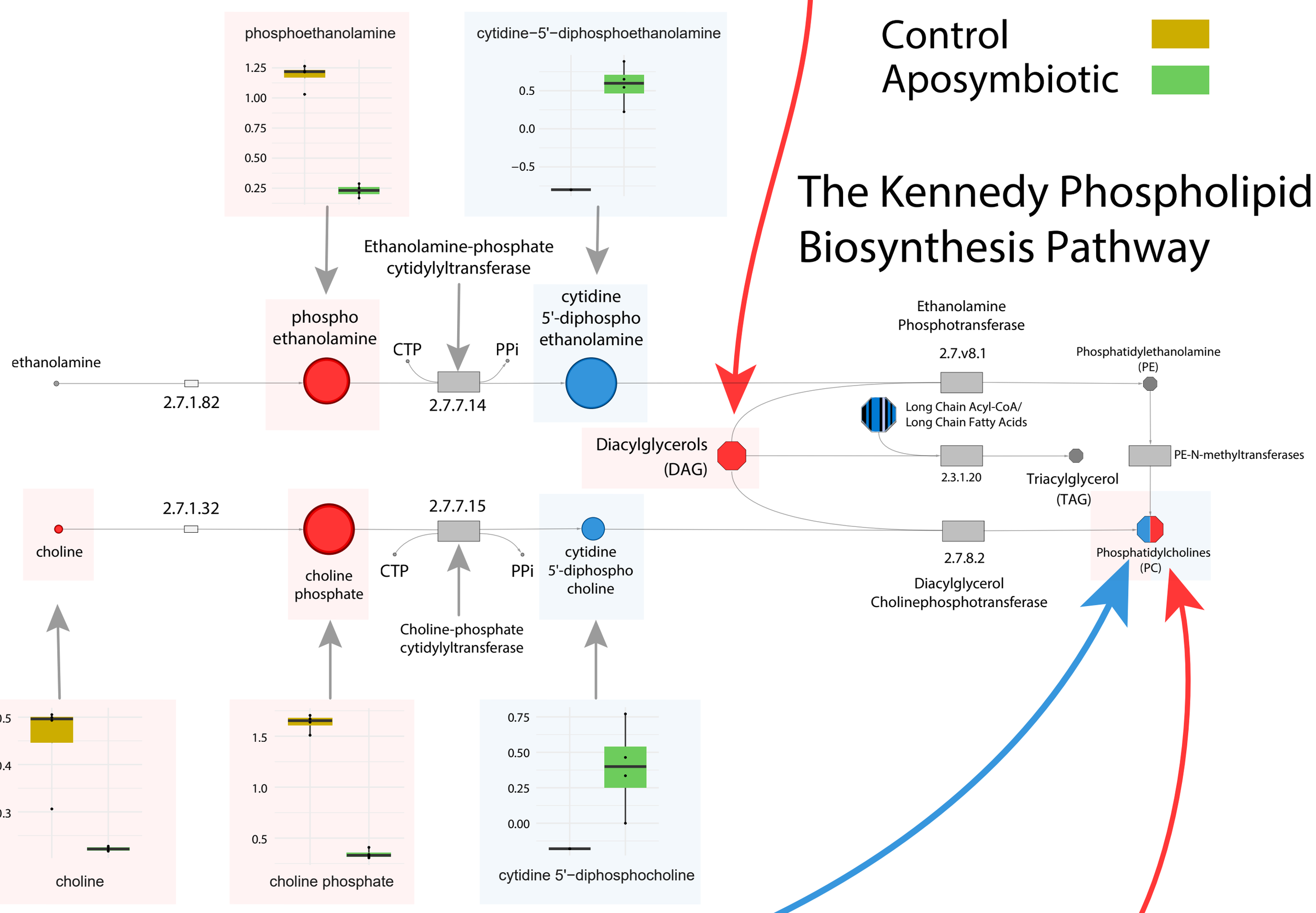
Diacylglycerols

Up



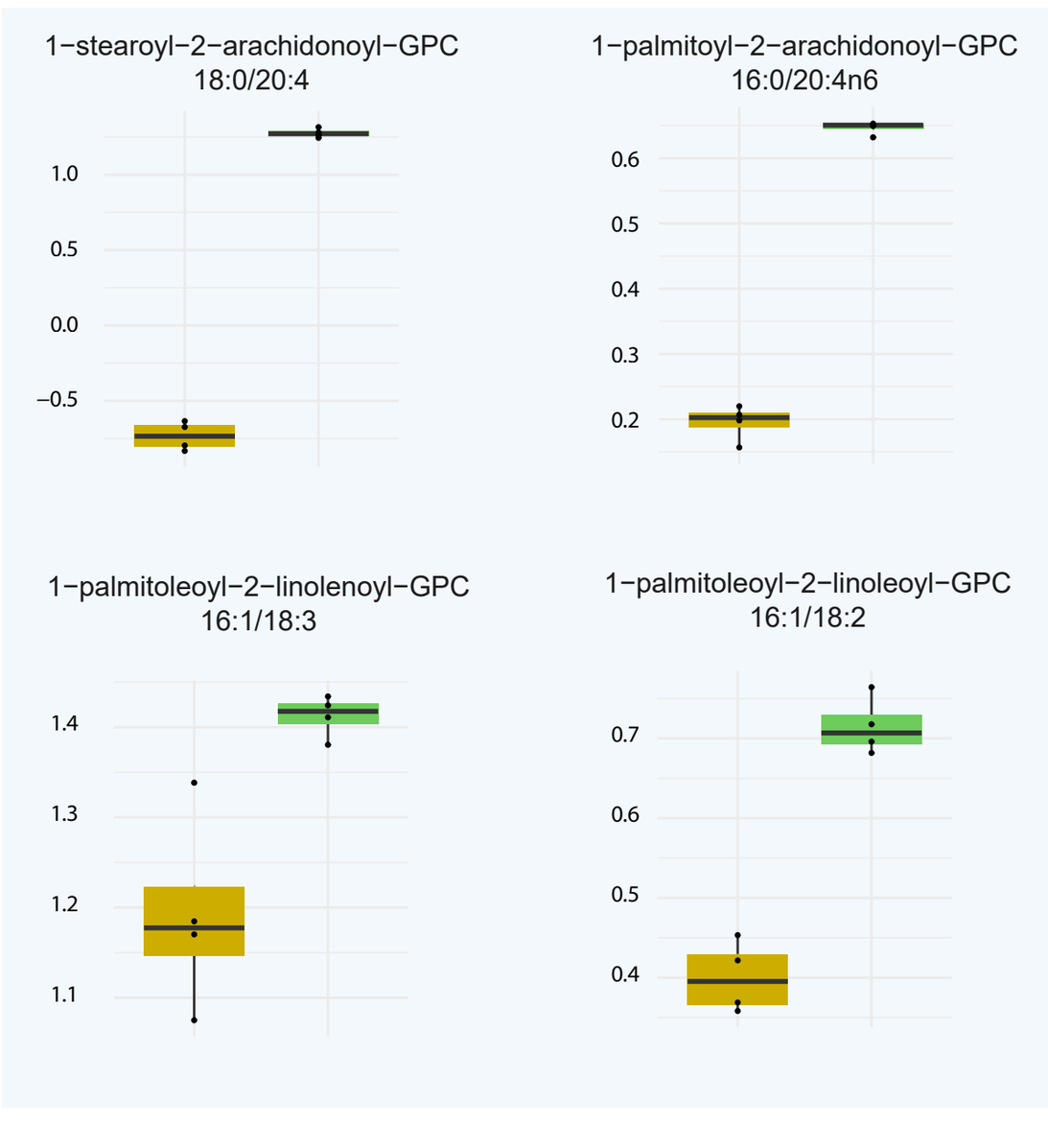
Down



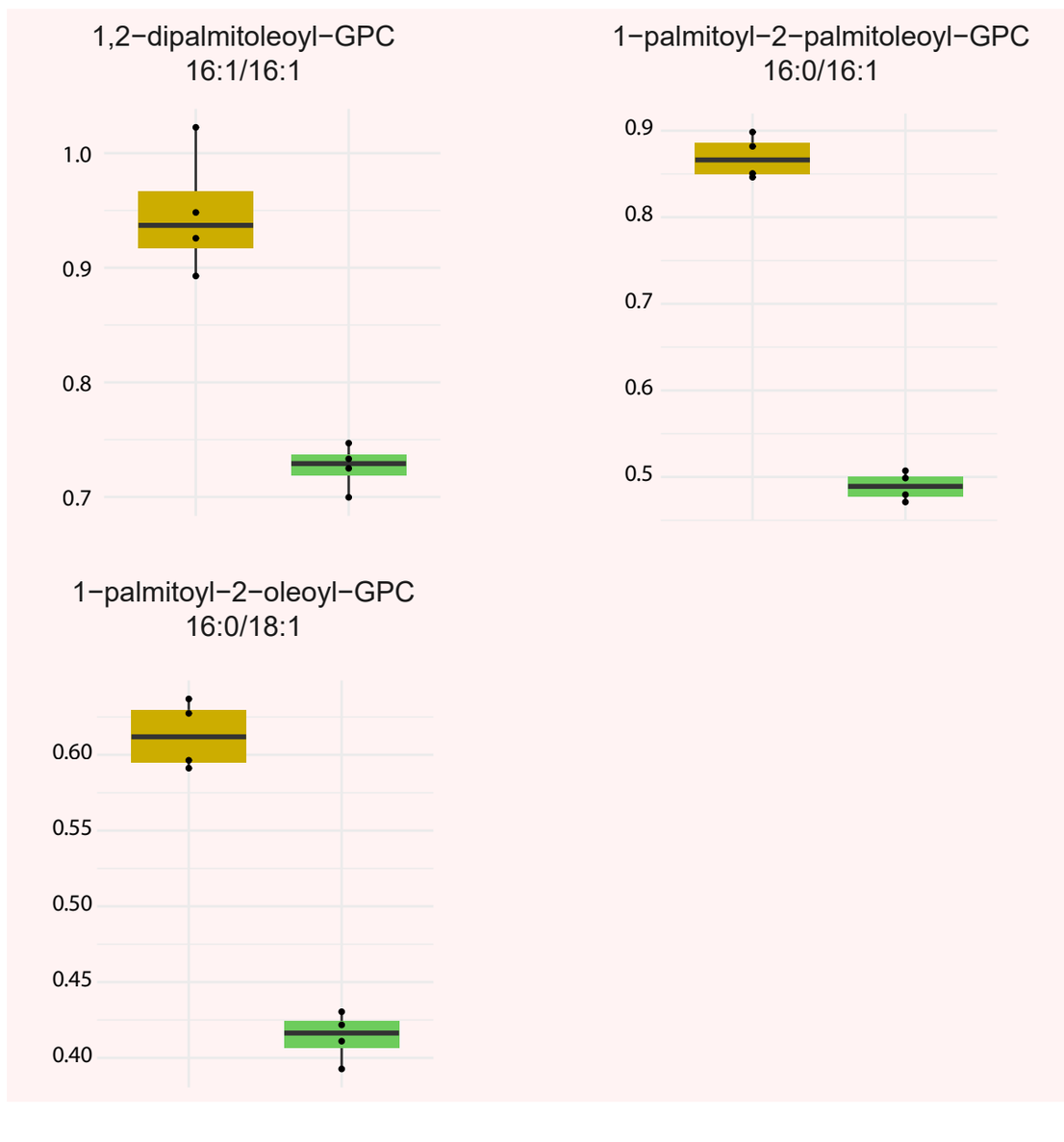


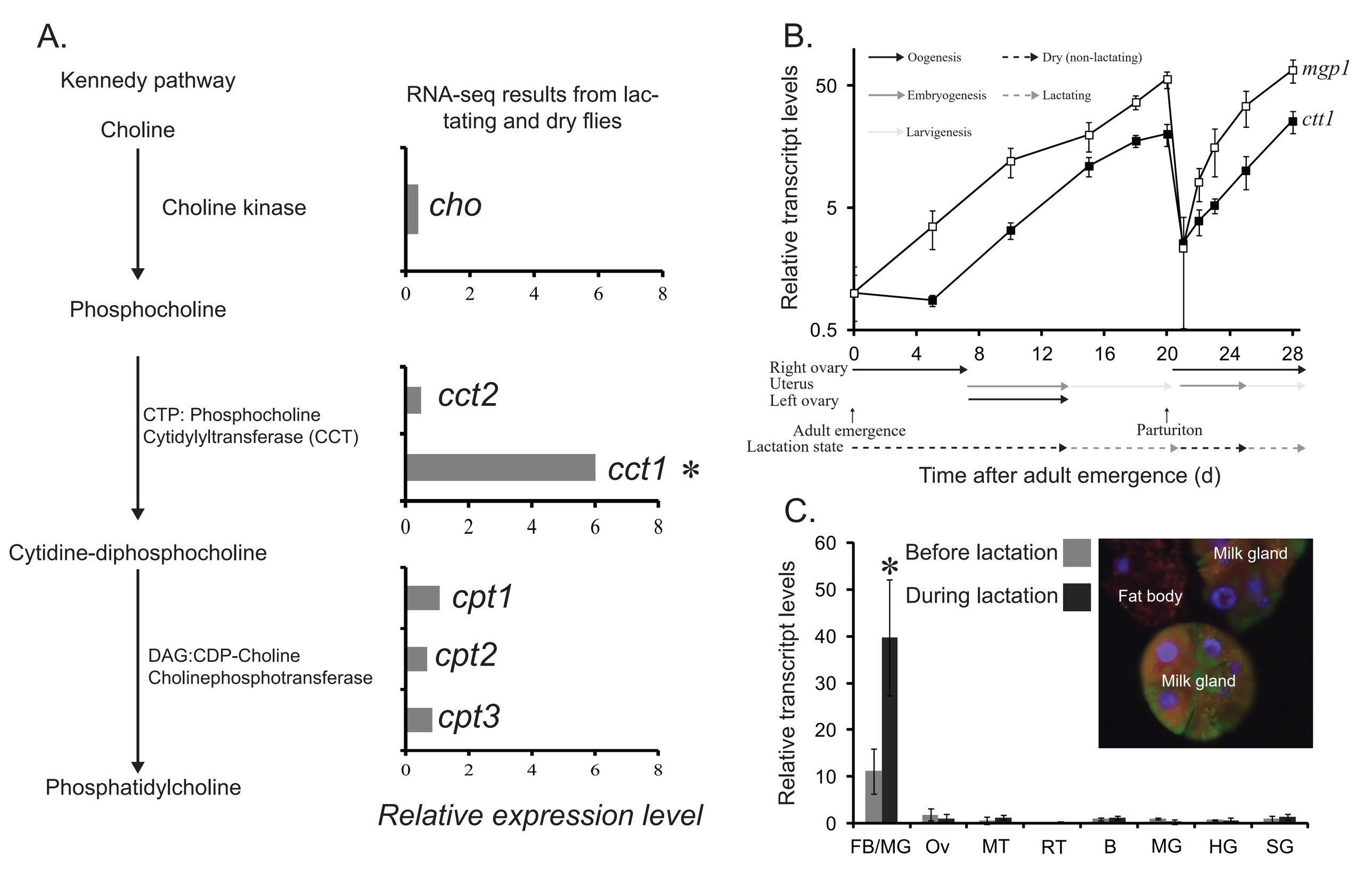
Phosphatidyl Cholines

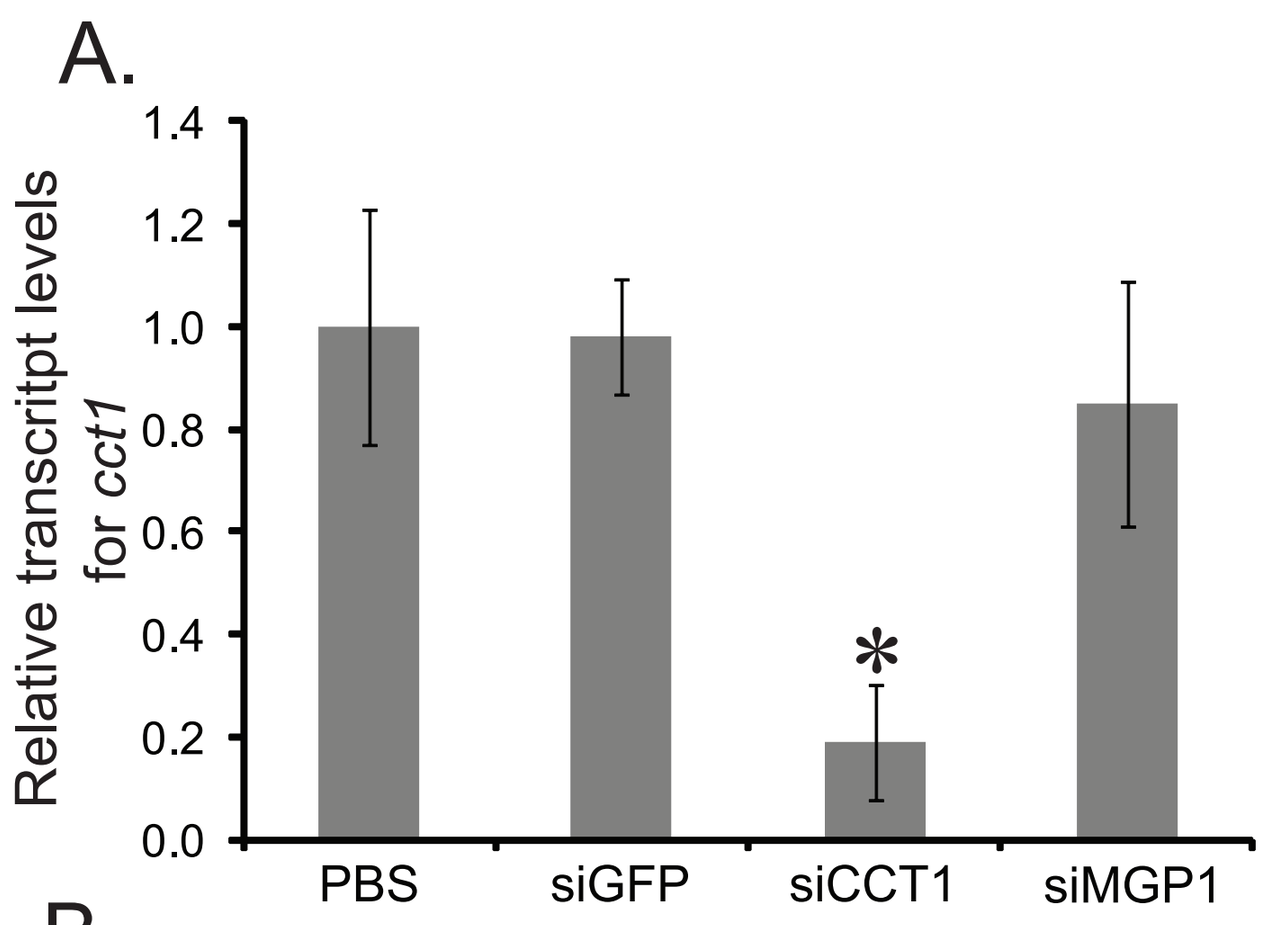
Up

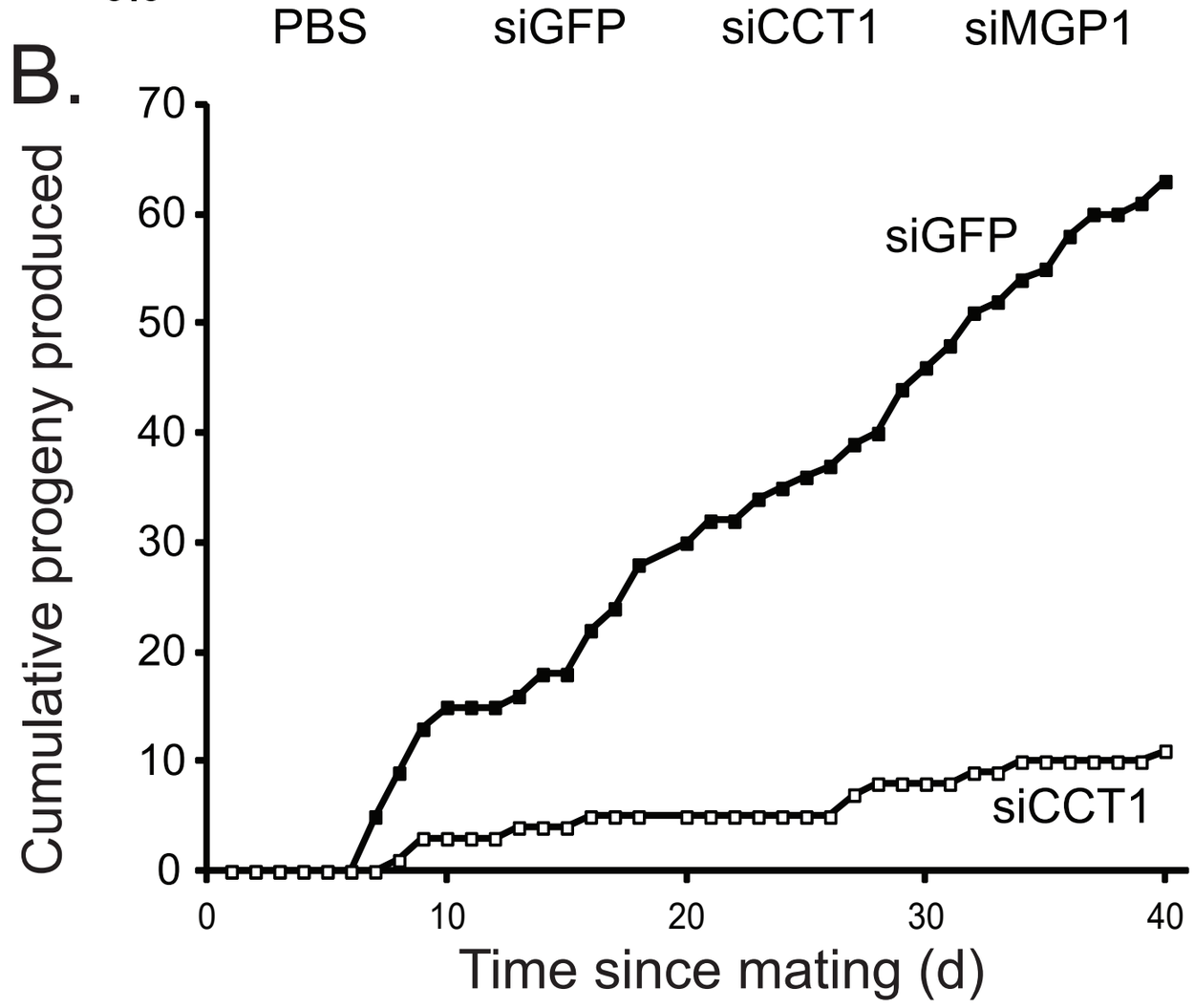


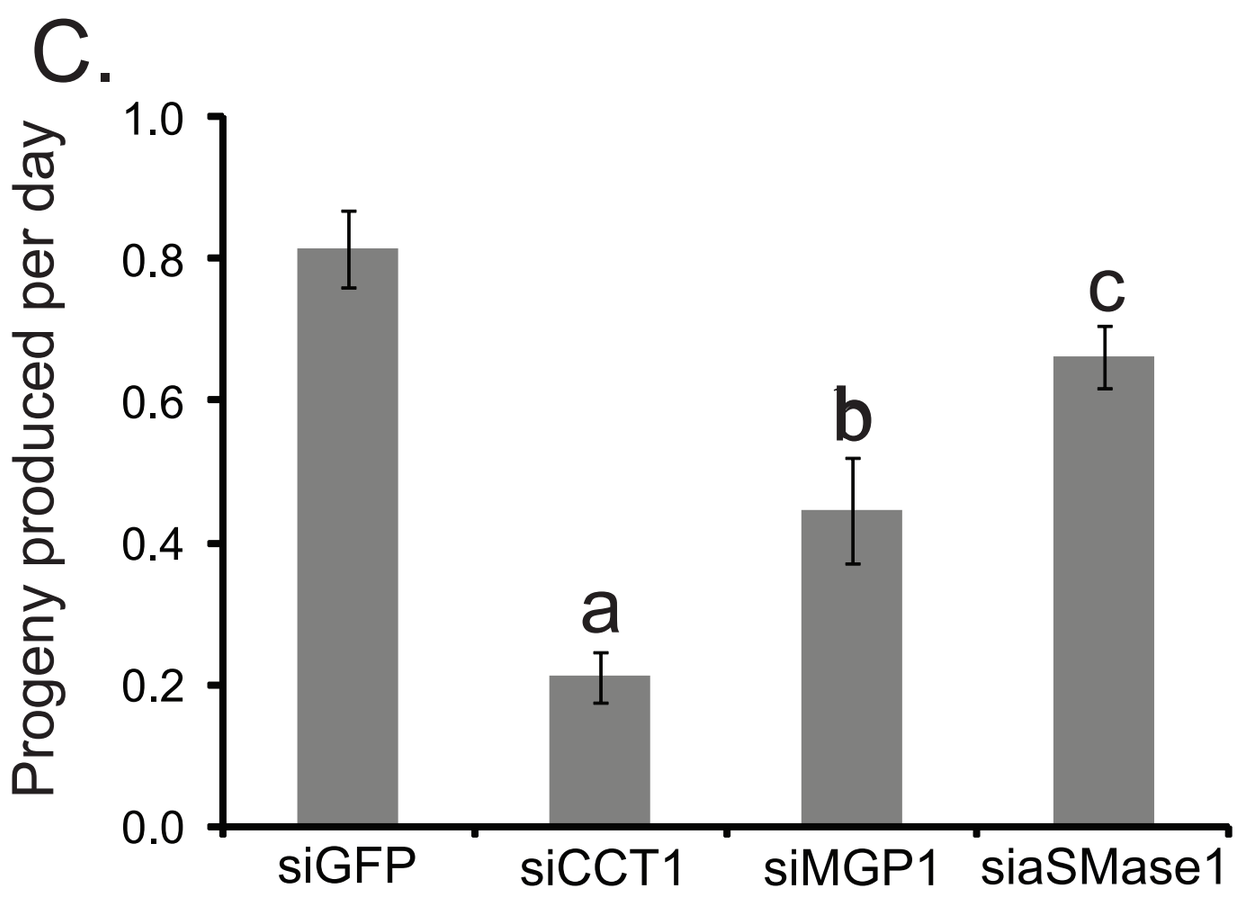
Down

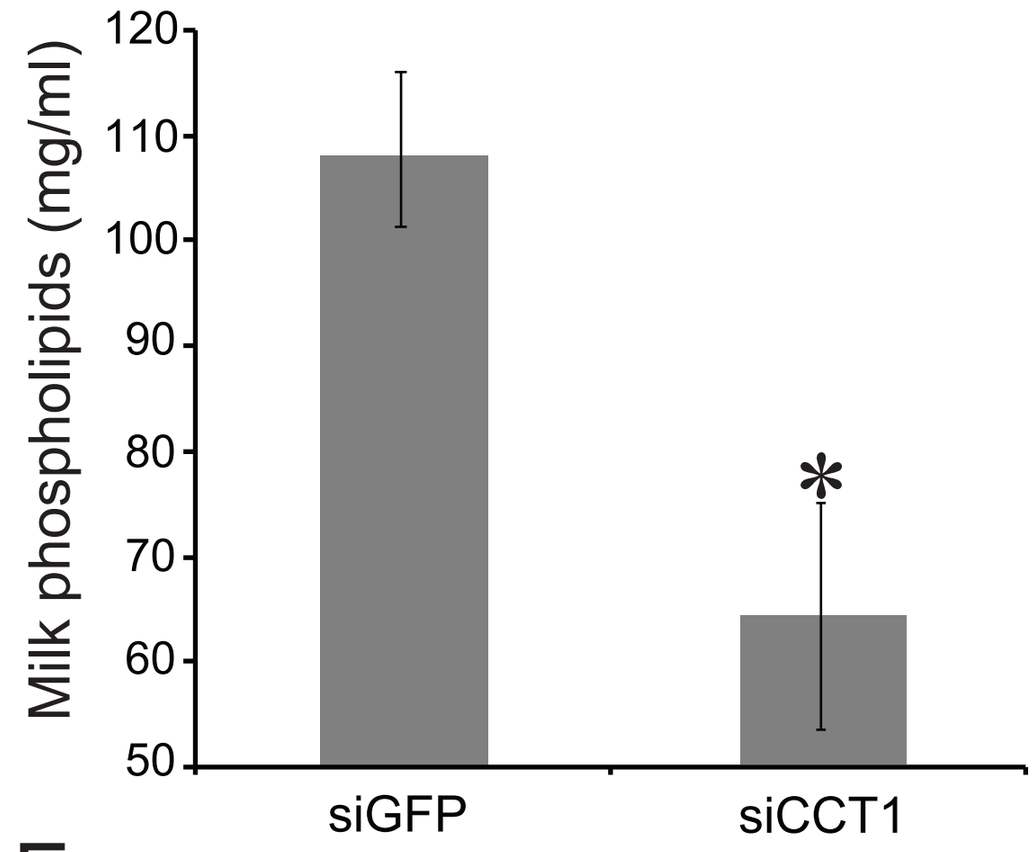


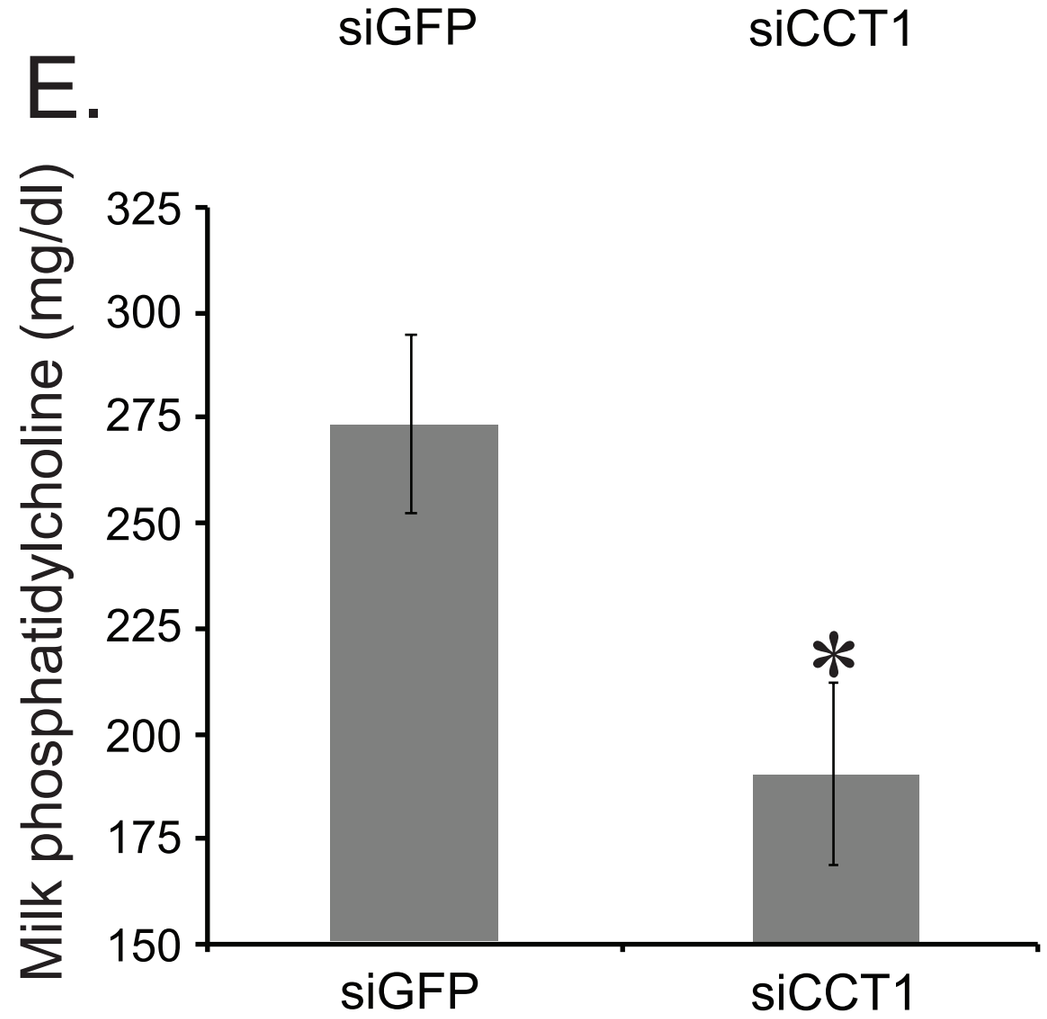

A.
Kennedy pathway
Choline
Choline kinase
Phosphocholine
CTP: Phosphocholine
Cytidylyltransferase (CCT)
Cytidine-diphosphocholine
DAG:CDP-Choline
Cholinephosphotransferase
Phosphatidylcholine
RNA-seq results from lactating and dry flies
cho
cct2
cct1 *
cpt1
cpt2
cpt3
0 2 4 6 8
Relative expression level
B.
Oogenesis
Dry (non-lactating)
Embryogenesis
Lactating
Larvigenesis
mgp1
ctt1
Relative transcritpt levels
50
5
0.5
0 4 8 12 16 20 24 28
Right ovary
Uterus
Left ovary
Adult emergence
Parturiton
Lactation state
Time after adult emergence (d)
C.
Before lactation
During lactation
*
Relative transcritpt levels
60 50 40 30 20 10 0
Milk gland
Fat body
Milk gland
FB/MG Ov MT RT B MG HG SG

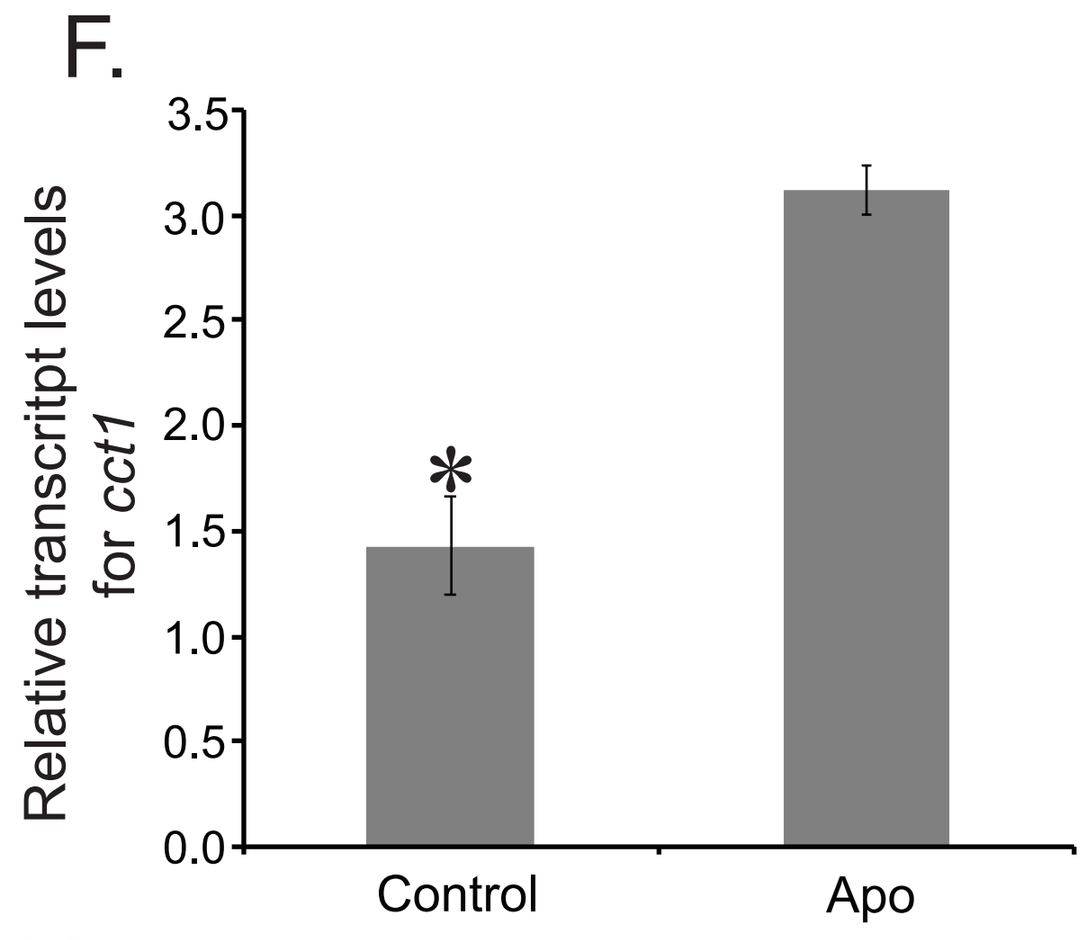

A.
Relative transcritpt levels for cct1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
PBS
siGFP
siCCT1
siMGP1

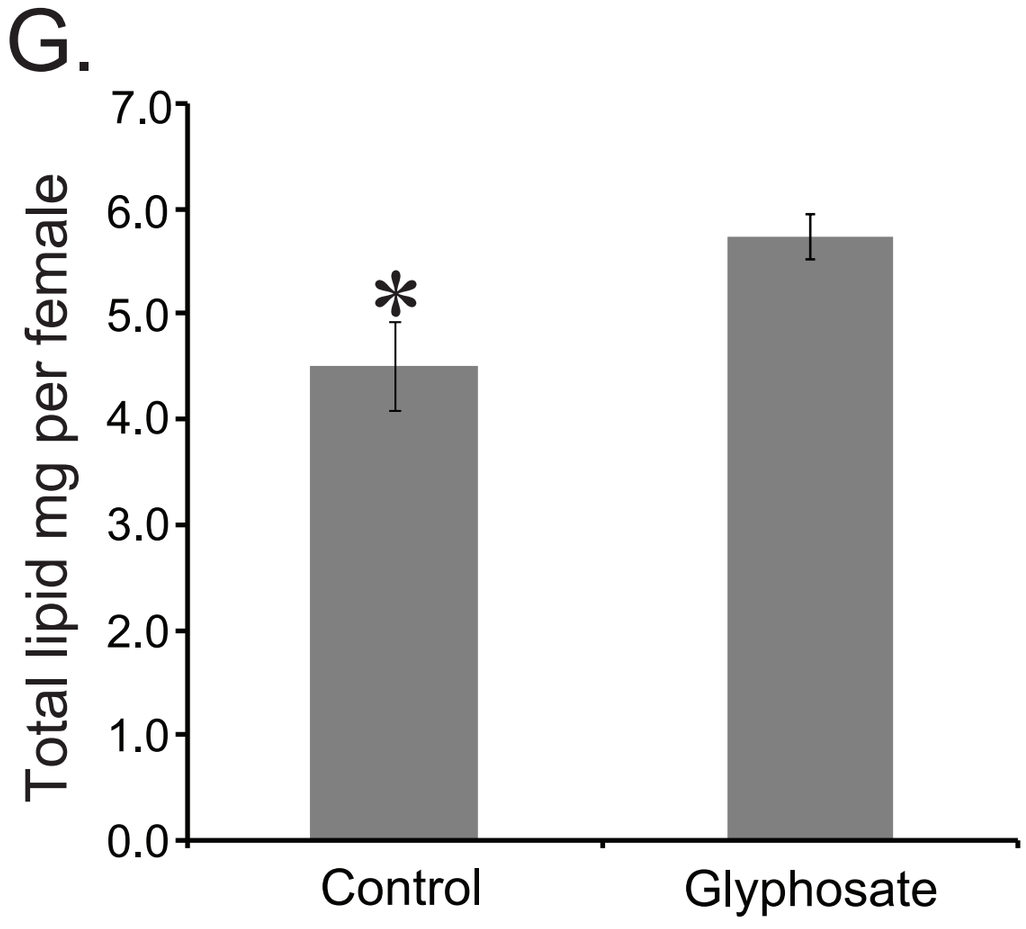

B.
Cumulative progeny produced
70
60
50
40
30
20
10
0
siGFP
siCCT1
0
10
20
30
40
Time since mating (d)


C.
Progeny produced per day
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
siGFP
siCCT1
siMGP1
siaSMase1


D.
Milk phospholipids (mg/ml)
120
110
100
90
80
70
60
50
*
siGFP
siCCT1


E.
Milk phosphatidylcholine (mg/dl)
325
300
275
250
225
200
175
150
*
siGFP
siCCT1


F.
Relative transcritpt levels for cct1
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Apo


G.
Total lipid mg per female
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
Control
Glyphosate

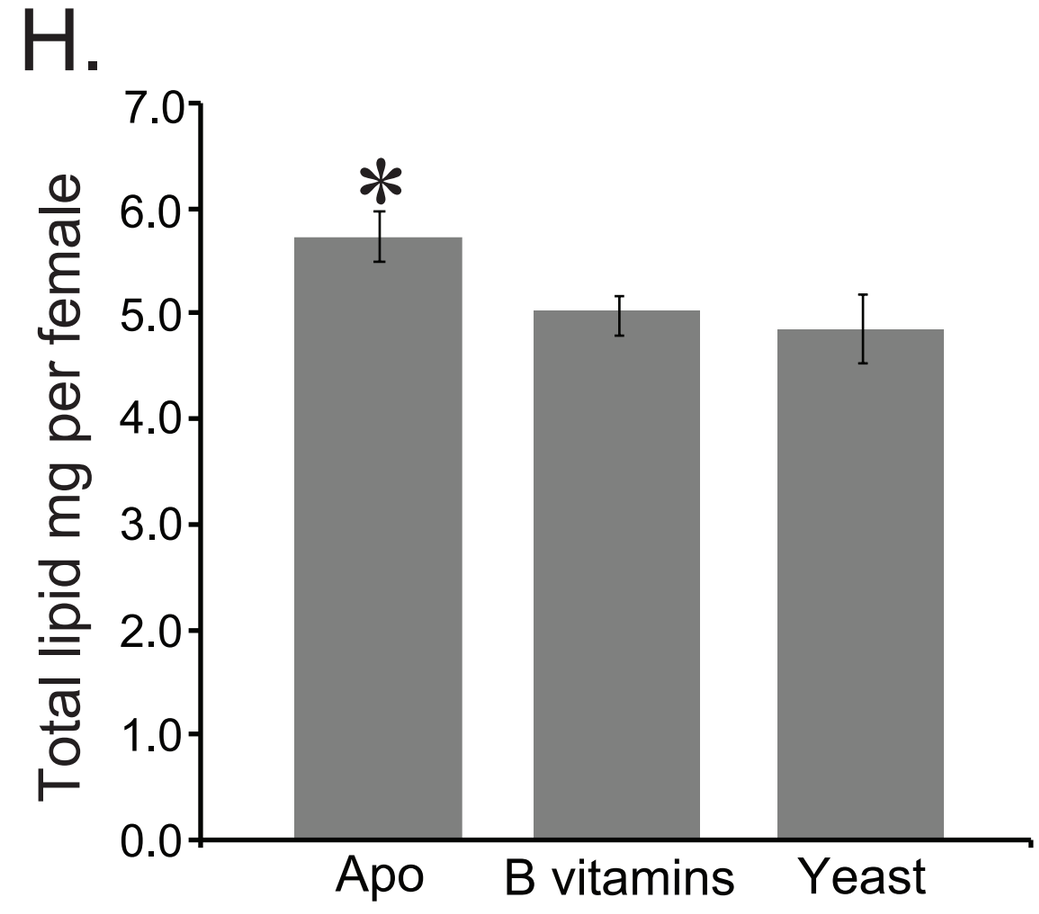
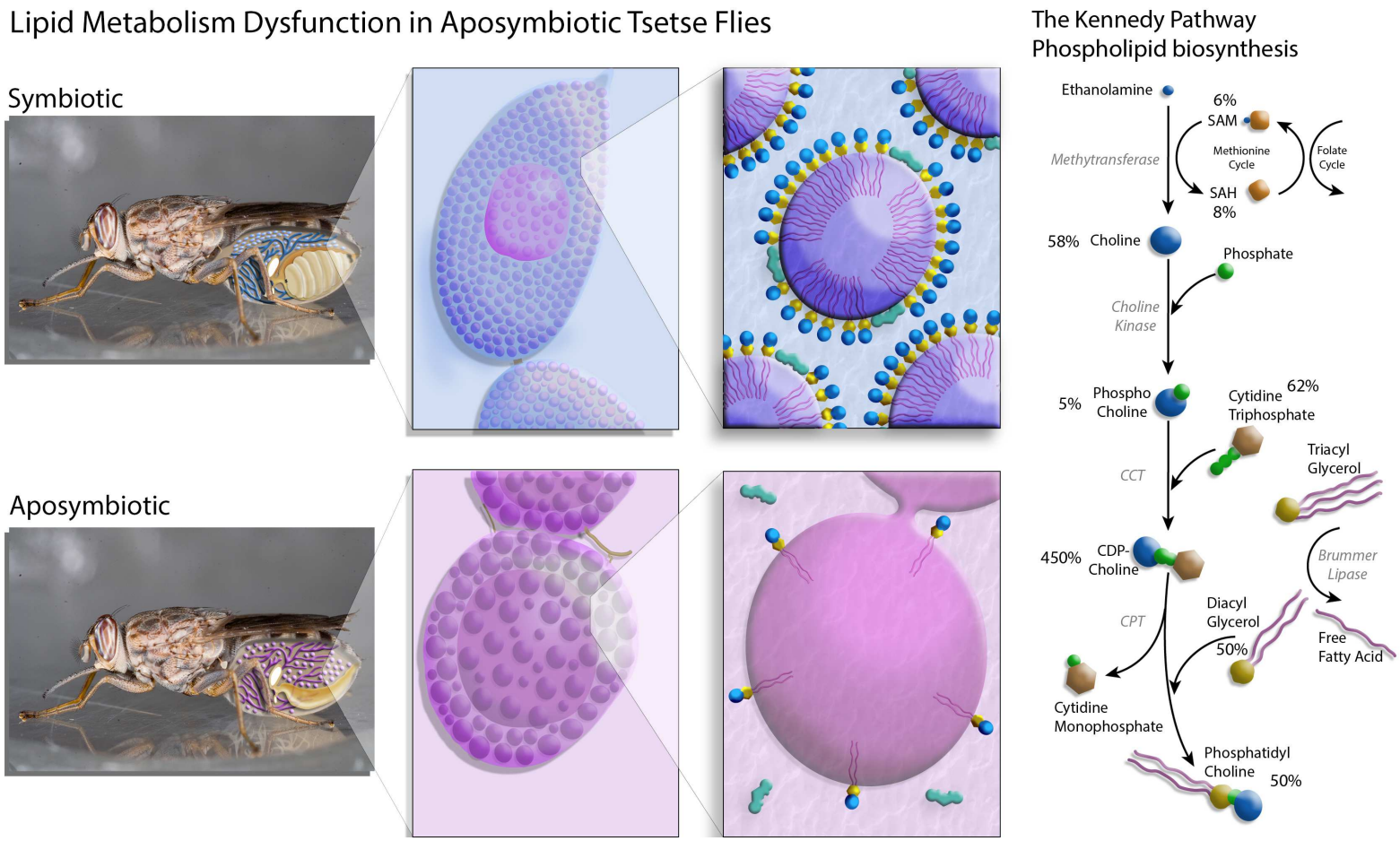

H.
Total lipid mg per female
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
Apo
B vitamins
Yeast

Figure 5


Lipid Metabolism Dysfunction in Aposymbiotic Tsetse Flies
Symbiotic
Aposymbiotic
The Kennedy Pathway
Phospholipid biosynthesis
Ethanolamine
6%
SAM
Methytransferase
Methionine Cycle
Folate Cycle
SAH
8%
58%
Choline
Phosphate
Choline Kinase
5%
Phospho Choline
Cytidine Triphosphate
62%
Triacyl Glycerol
CCT
450%
CDP-Choline
Brummer Lipase
CPT
Diacyl Glycerol
50%
Free Fatty Acid
Cytidine Monophosphate
Phosphatidyl Choline
50%