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Article

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Lipid Metabolism Dysfunction in Aposymbiotic Tsetse Flies
Symbiotic
Aposymbiotic
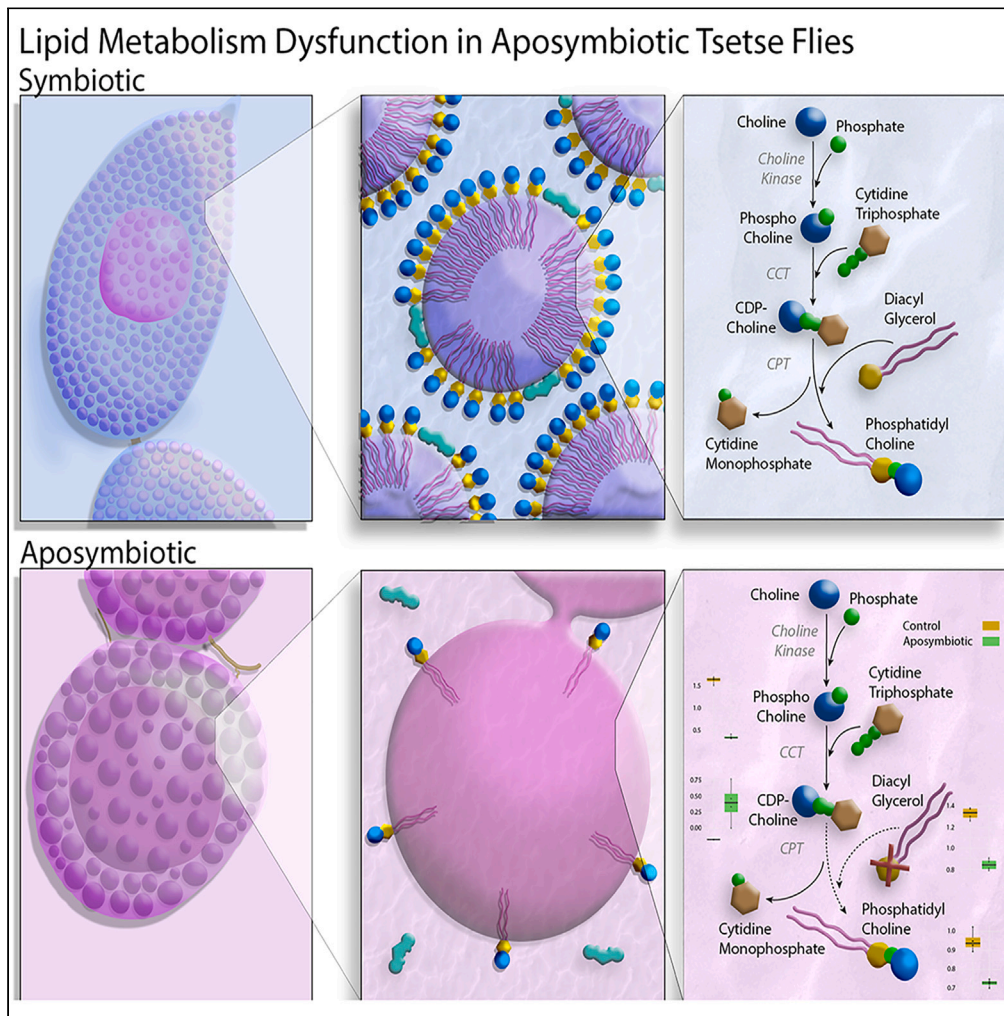
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Highlights

Tsetse flies have increased phosphatidylcholine biosynthesis during pregnancy

Removal of symbionts alters phosphatidylcholine biosynthesis and induces abortion

Reduced phosphatidylcholine induces abortion and yields obese mothers

B vitamins from symbionts are required for lipid metabolism during pregnancy

Article

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

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SUMMARY

Lipid metabolism is critical for insect reproduction, especially for species that invest heavily in the early developmental stages of their offspring. The role of symbiotic bacteria during this process is understudied but likely essential. We examined the role of lipid metabolism during 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, which is critical for phosphatidylcholine biosynthesis in the Kennedy pathway. Experimental removal of *Wigglesworthia* impaired lipid metabolism via disruption of the Kennedy pathway, yielding obese mothers whose developing progeny 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 the proper function of both lipid biosynthesis and lipolysis to maintain tsetse fly fecundity.

INTRODUCTION

Reproduction represents an evolutionarily imperative and metabolically taxing physiological process for all animals.[1,2] This may be especially true for insects, which maintain a high reproductive rate, even though progeny quality declines throughout adulthood.[3,4] One such insect is the tsetse fly, *Glossina spp.*, which reproduces via a process called "adenotrophic viviparity" (gland-fed, live birth[5]). 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 (MGs). Thus, female tsetse flies supply all the nutrients necessary to support their progeny's embryonic and larval development. Tsetse milk consists of 20–30 mg of carbohydrates, lipids, and proteins in an aqueous base.[5–8] The rapid incorporation of nutrients into milk products during lactation reduces total maternal lipid and protein content by nearly 50% and 25%, respectively.[9] Following birth, the MGs rapidly involute, and lipid reserves accumulate to support a subsequent offspring.[9–11] Tsetse mothers continuously endure this metabolically intense K-selected reproductive strategy through their adulthood, ultimately birthing up to 8–12 larvae per lifetime.[3,4]

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 to support the metabolically intense process of milk production. To overcome this, tsetse flies have developed intimate, long-term associations with a symbiotic bacterium that supplements specific micronutrients missing or present in low quantities in host blood. This bacterium is the maternally transmitted obligate endosymbiont *Wigglesworthia glossinidia*, which has been associated exclusively with tsetse species for 50–80 million years.[12] These bacteria reside only in a specific organ (bacteriome) attached to the gut or in the MG to move to developing offspring. As a result, *Wigglesworthia* has experienced massive genome erosion.[13,14] However, the bacterium retains genes required to produce a variety of B vitamins that are largely absent from vertebrate blood.[14,15] The metabolism of proline, which circulates in tsetse hemolymph and replaces carbohydrates as the flies' prominent energy source, is fueled by B vitamins generated by *Wigglesworthia*.[16] Accordingly, antibiotic-mediated clearance of *Wigglesworthia* results in a dramatic reduction in multiple B vitamin-associated compounds that serve as essential cofactors

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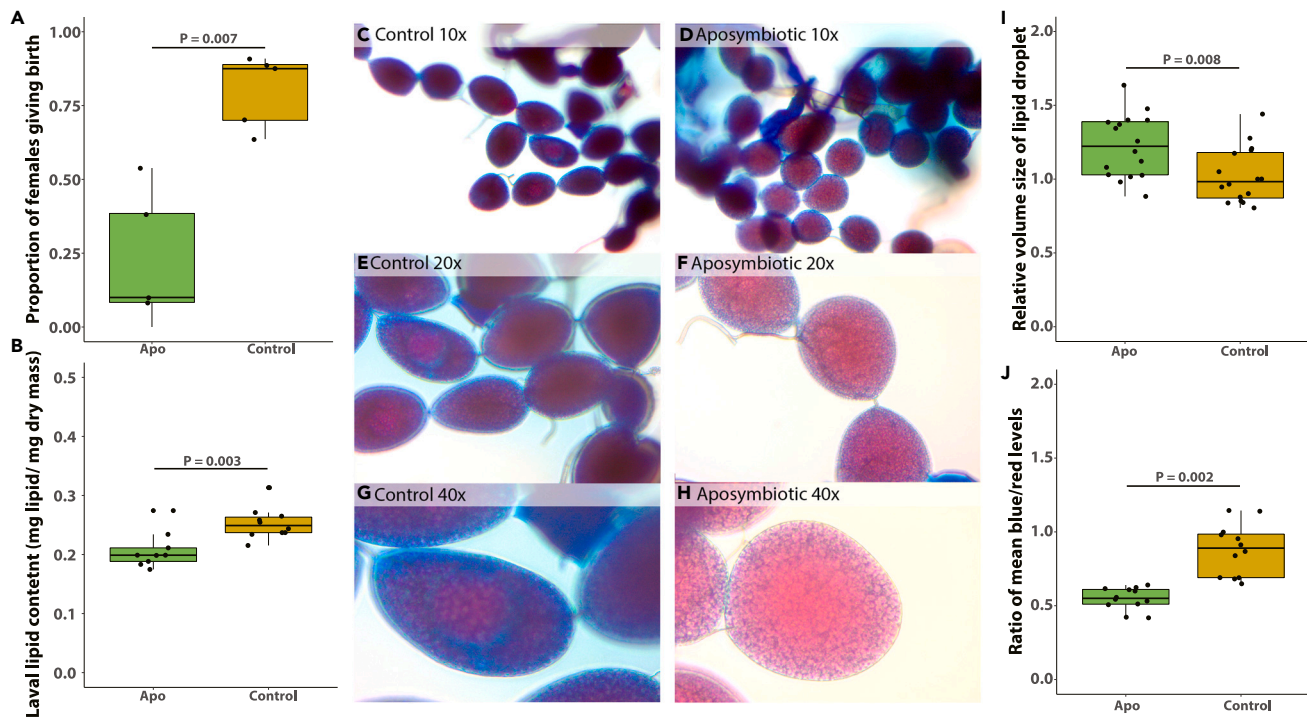
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Figure 1. Reproduction and lipid metabolism is dysfunctional in aposymbiotic flies

Proportion of females giving birth

(A) N = 4, and lipid content in the surviving larvae (B) are significantly reduced in aposymbiotic flies (t test, p value 0.007 and 0.0003, respectively). N = 8–10.

(C–H) Nile blue staining reveals significant differences in the neutral (pink) and charged lipid (blue) composition in aposymbiotic and control flies.

(I) Volume of the fat body is increased significantly in aposymbiotic flies (t test, p value 0.008). N = 12–14.

(J) Image quantification reveals significantly higher charged lipids in the control compared to the aposymbiotic flies (t test, P value 0.002). N = 12–14.

in fundamental metabolic pathways, including the pentose phosphate pathway, nucleotide biosynthesis, methionine/cysteine metabolism, and amino acid and lipid metabolism. These deficiencies yield adult female flies incapable of milk production, resulting in abortion of early instar larvae.[15,17,18] However, the mechanistic basis for this phenotype remains unclear. Other bacteria have been associated with tsetse flies,[19–21] but these are only at low levels. *Sodalis* is a commonly found facultative bacteria for tsetse flies but is not present in all lineages,[19–21] highlighting that *Wigglesworthia* mediates nutritional supplementation.

Here we examined the interplay among obligate symbionts, lipid metabolism, and reproductive output of tsetse flies. The maternal transfer of nutrients to developing offspring is an energetically demanding process for all insects. However, tsetse flies, similar to other viviparous systems, invest even more into their progeny by providing resources for embryonic and larval development. This study demonstrates that critical symbiont-derived factors promote phospholipid (PL) synthesis, facilitating rapid lipolysis of stored lipid reserves to nourish the developing intrauterine progeny. Other insects that rely on symbiont-derived B vitamins, including bed bugs and lice,[22–25] are likely to experience impaired lipolysis and reduced fecundity without their symbiotic partner(s) as the molecular mechanisms underlying this process are not unique to *Glossina*.[26] Furthermore, similarities in live birth across animal systems indicate that micronutrient deprivation is likely to significantly impact metabolic processes with similar negative consequences that extend from invertebrates to vertebrates.[27]

RESULTS

Aposymbiotic flies are obese and fail to reproduce

Antibiotic-treated, endosymbiont-free (aposymbiotic) tsetse flies failed to reproduce successfully (Figure 1A), as previously shown.[16–18] When early instar larvae from aposymbiotic mothers were removed before abortion and compared to control larvae, their lipid levels were 25–30% lower, indicating substantially reduced lipid transfer from mother to larvae in the aposymbiotic state (Figure 1B). Aposymbiotic

Diacylglycerols
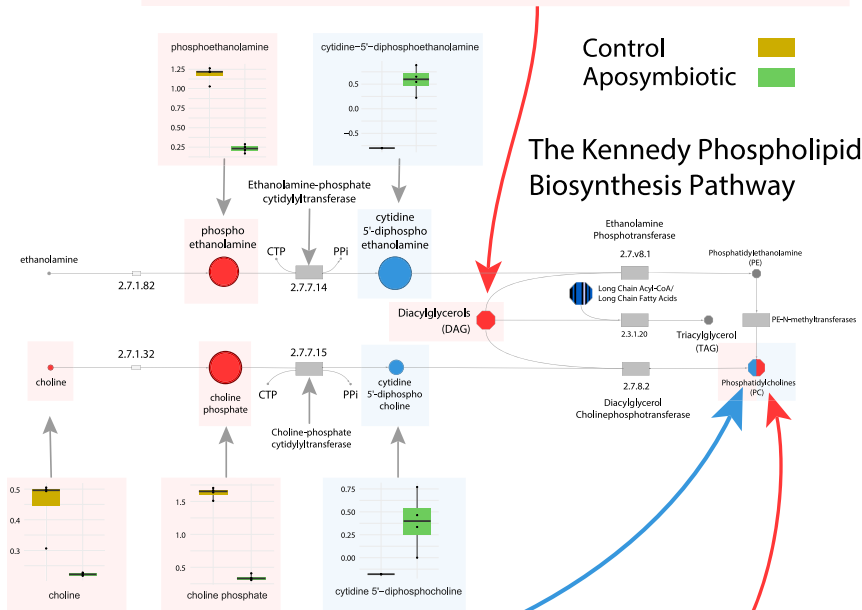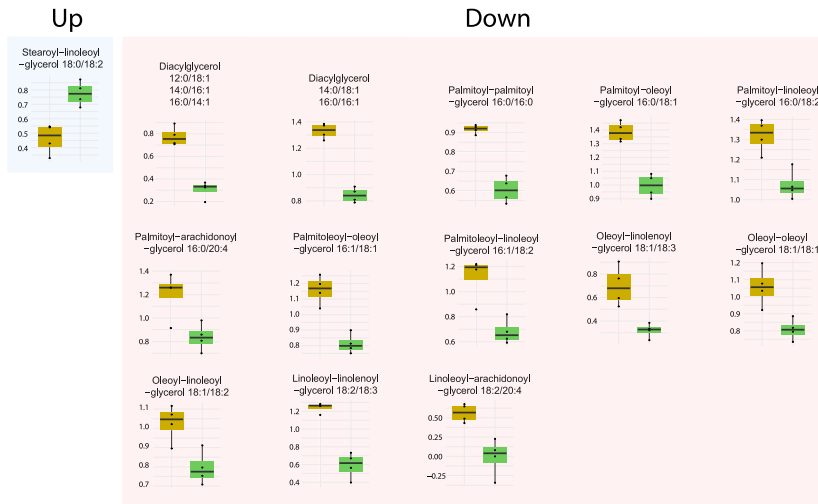
The Kennedy Phospholipid Biosynthesis Pathway

Control / Aposymbiotic

Phosphatidyl Cholines
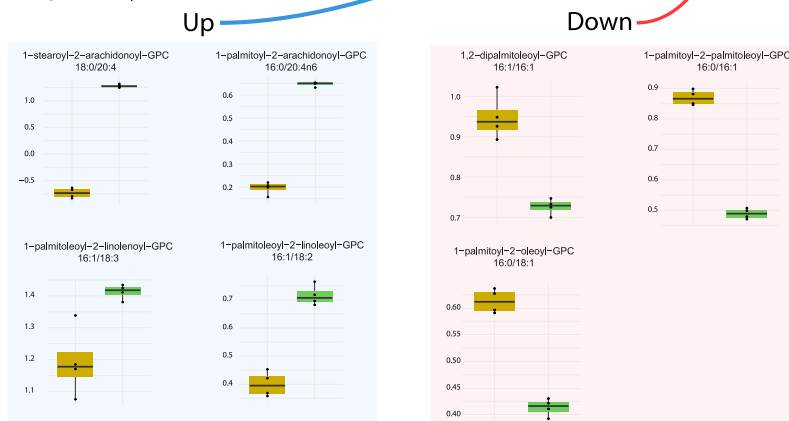
Figure 2. Lipidomics reveals a significant shift in lipid moeities following symbiont removal

Compounds showing a significantly different fold change in their abundance in control (yellow) and aposymbiotic (green) flies are shown. Red denotes increase in compounds of the Kennedy pathway in aposymbiotic flies, while blue indicates lipids showing higher fold change in control individuals. Specifically, there is a major dysfunction in the Kennedy pathway for phosphatidylcholine synthesis. N = 5, * indicates significance based on a t test. Details of specific results are shown in Figures S1–S6.

females show higher total lipid levels, with an average of 5.66 ± 0.44 mg lipids per female to 4.85 ± 0.34 in symbiotic females. This phenotype was associated with increased lipid droplet (LD) size in the fat body (FB, Figures 1C–1H), suggesting defect(s) in lipolysis. Additionally, Nile blue staining of FB tissue revealed a reduction in charged PLs relative to neutral storage lipid species, including tri- and diglycerides, in aposymbiotic flies relative to control flies (Figures 1I and 1J). These initial studies suggest a major dysfunction in lipid metabolism following symbiont clearance.

Lipidomics reveals massive shifts in metabolic lipid processing

To understand the biochemical landscape facilitating the accumulation of triacylglycerols in endosymbiont-free flies, we generated aposymbiotic females via tetracycline application. We analyzed pooled FB and MG tissues, comparing aposymbiotic and untreated (control) age-matched females. The differential biochemical composition analysis revealed substantial alterations in multiple biochemical categories in aposymbiotic flies, including B vitamins, creatine/creatinine, amino acids, diacylglycerols (DAGs), PLs, sphingolipids, lysolipids, and polyunsaturated fatty acids (Figures S1–S6). These changes yield an overall composition difference in the lipid profiles between control and aposymbiotic flies (Figure S7, ANOSIM, R = 0.879, p = 1.03E-06).

Many of the observed changes are via either direct or indirect effects of dysregulation of PL biosynthesis via the Kennedy pathway (Figure 2). Notably, aposymbiotic flies were deficient in key precursors to PL synthesis, including choline, phosphocholine, the majority of the detected DAGs, and phosphatidylcholines (PCs). Many DAG variants are present in adult female tsetse, and they differ in acyl chain lengths and desaturation state. The most abundant DAGs in control flies contain 16/18 carbon unsaturated or mono-unsaturated side chains (Figures 2 and S2). In contrast, aposymbiotic flies show a broad reduction in the abundance of these DAGs. Among PCs, aposymbiotic flies generally lack moieties containing 16/18 carbon unsaturated or mono-unsaturated side chains. In contrast, those containing 18/20 carbon polyunsaturated fatty acids are abundant (Figure S1). This may reflect the observed overabundance of polyunsaturated free fatty acids in aposymbiotic flies (Figure S4). The substantial deficiencies in the precursor molecules required for PC production suggest that *Wigglesworthia*-derived factors either directly or indirectly facilitate the production of PL precursors. Aposymbiotic flies also demonstrate a profound deficiency in creatine and creatinine (Figure S6). Creatine/creatinine biosynthesis and the urea cycle depend on folate (vitamin B9), which is provided to tsetse flies by *Wigglesworthia*.[28–30] Components of the urea cycle are also reduced in abundance in aposymbiotic flies.

Choline-phosphate cytidylyltransferase 1 (*cct1*) represents a critical enzyme during tsetse fly reproduction

Prior RNA sequencing (RNA-seq) gene expression analyses during tsetse pregnancy and milk production revealed a suite of highly expressed milk protein-encoding genes.[7,31,32] Notably, transcripts for the enzyme choline-phosphate *cct1* were upregulated similarly to milk proteins in pregnant flies (Figure 3A). This enzyme participates in PC synthesis. PCs are critical for regulating LD size and formation to facilitate lipolysis during energy-intensive processes.[33] The expression of *cct1* decreases immediately post-parturition and increases again during MG involution following parturition (Figure 3B). Tissue-specific expression analysis confirmed *cct1* transcripts were abundant in the FB and MG. In support of this finding, *in situ* hybridization revealed *cct1* expression in both of these tissues (Figure 3C). These data suggest that *cct1* is critical for lipid metabolism during tsetse pregnancy, likely functioning in the FB and MG, to break down lipids critical for milk production, or directly in milk LD production.

Suppression of *cct1* phenocopies the aposymbiotic state

To confirm if impaired *cct1* function and altered PL metabolism perturb lipid metabolism during viviparity, we suppressed *cct1* expression using RNA interference (RNAi) (Figure 4A). Similar to aposymbiotic flies, *cct1* knockdown flies produce few progeny (Figures 4B and 4C). We noted a massive decrease in levels
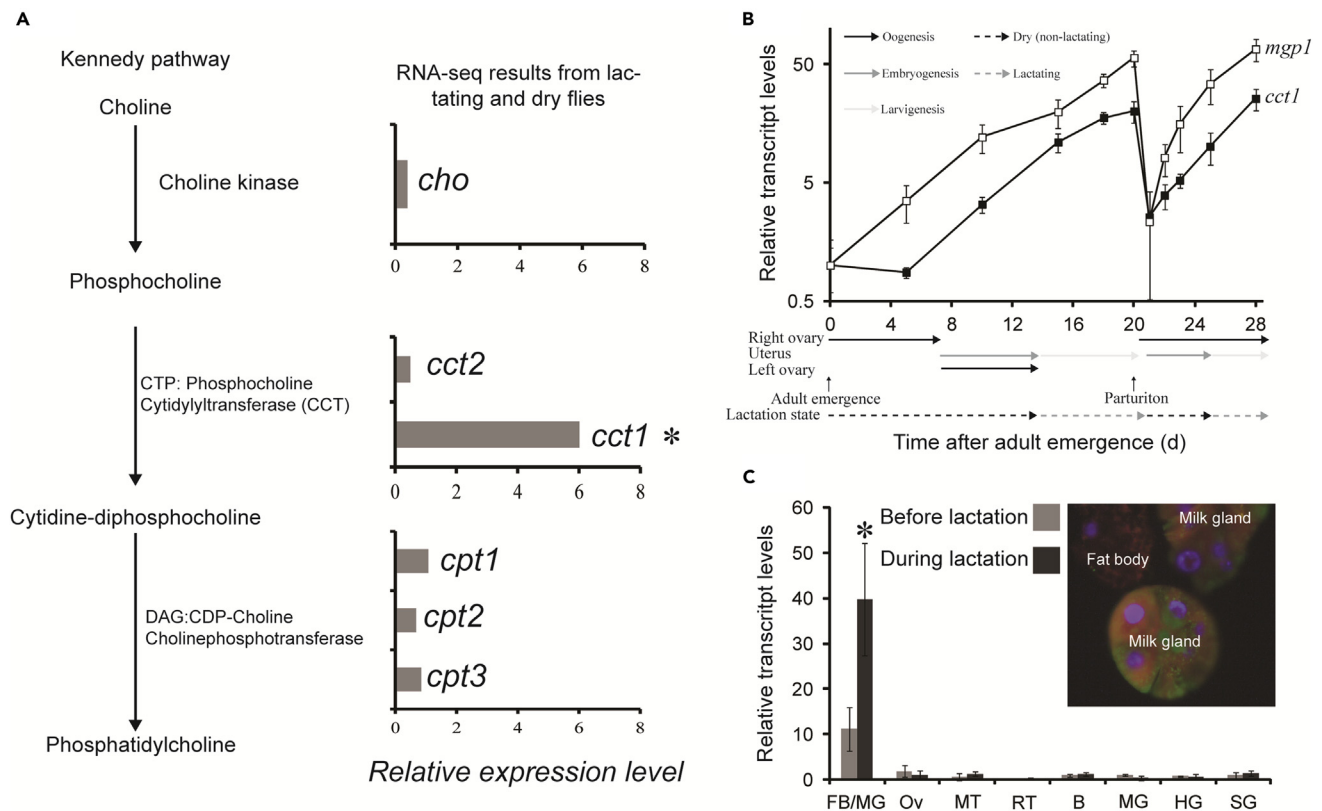
Figure 3. Increased expression of *choline-phosphate cytidylyltransferase 1 (ctt1)* is associated with tsetse fly pregnancy

(A) Previous RNA-seq studies[7,31] reveal that *cct1* is expressed in pregnant/lactating flies.

(B) qPCR indicated that the expression pattern for *ctt1* is correlated with the pregnancy cycle, similar to a milk protein critical to feed the developing larvae (*milk gland protein 1, mgp1*). N = 4.

(C) Expression of *cct1* is localized in both the milk glands and fat body, suggesting critical roles in lipid breakdown and milk production. N = 4, *, denotes significance based on a Kal's proportion test or t test. Inset - *cct1 in situ* hybridization, red, along with milk gland protein (MGP) immunohistochemistry, green, and DAPI staining of nuclei, blue, of a cross section of milk gland tubules and fat body. Negative controls not treated with Digoxigenin-labeled sense RNA probes displayed no signal.

of PLs and PC in developing larvae, indicating that *cct1* is necessary to ensure sufficient lipid transfer to the developing larva (Figures 4D and 4E). This decrease likely accounts for the 31% reduction in total lipids measured from larvae deposited from *cct1* knockdown flies (0.20 ± 0.03 mg lipid/mg dry mass for *cct1* RNAi vs. 0.29 ± 0.04 for control). In aposymbiotic females, *cct1* expression increases substantially (Figure 4F), perhaps as a compensatory response to reduced PL levels required for lipid transfer during pregnancy. We confirmed that *cct1* suppression did not alter the levels of vitamin B6 (780 ± 0.22 nmol/L for siGFP (green flourescent protein) and 801 ± 0.42 nmol/L for siCCT1), which is expected as the B vitamin-related issues are not expected to be impacted by the levels of CCT1.

Tetracycline treatment impairs mitochondrial function,[34] which could represent an off-target outcome that significantly impacts tsetse lipid metabolism. As such, we treated flies with glyphosate, which specifically inhibits *Wigglesworthia* folate production.[35] This treatment yielded flies that presented obesity measures similar to their aposymbiotic counterparts. This finding confirmed that *Wigglesworthia*-derived folate acts as an essential cofactor for tsetse PL metabolism processes (Figure 4G). In addition, supplementation of dietary blood with *Wigglesworthia*-containing bacteriome homogenate or yeast did reduce obesity (Figure 4H). This confirms that symbiont-derived products are likely critical to lipid metabolism.

DISCUSSION

This study defines a novel feature of host-symbiont dynamics in tsetse showing dysfunctional PL metabolism as a resultant phenotype of symbiont elimination (Figure 5). Tsetse fly pregnancy requires a rapid
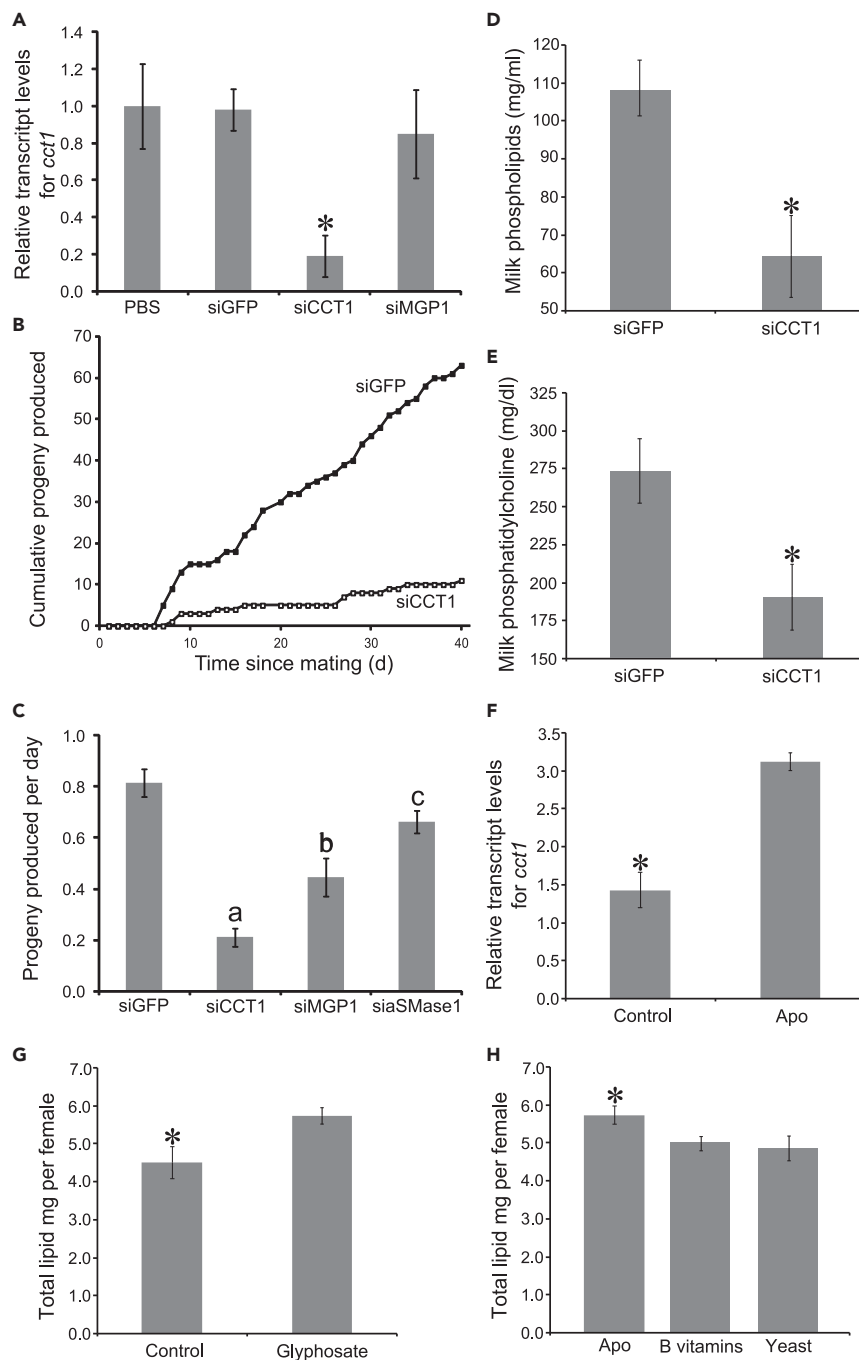
Figure 4. Suppression of *cct1* produces aposymbiotic phenotypes of altered lipid metabolism during pregnancy

(A) RNA interference reduces the expression of *cct1*. N = 5, *, denotes significance based on a t test. *mgp1, milk gland protein 1*. GFP, green flourescent protein. Primers listed in Table S1.

(B and C) Total progeny production and progeny generated per day are reduced following suppression of *cct1*. N = 3 groups of 10. Different letters denote significance based on an ANOVA. *acid smase1*, *asmase1*.

(D and E) Milk phospholipids and phosphatidylcholine are reduced when *cct1* is reduced. N = 5. *, denotes significance based on a t test.

(F) Aposymbiotic flies show increased expression of *cct1* as a potential compensatory mechanism to allow for lipolysis during pregnancy. N = 6. *, denotes significance based on a t test.
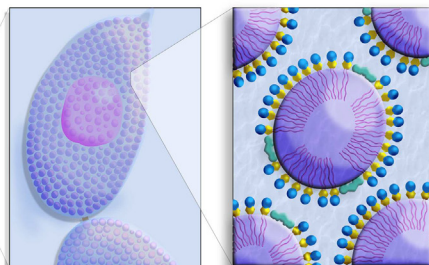
(G) Glyphosate treatment increased lipid levels within flies. N = 10. *, denotes significance based on a t test.

(H) Supplementation of the bloodmeals with yeast extract or *Wigglesworthia*-containing bacteriome extract prevented the obese phenotype in aposymbiotic flies. N = 10. *, denotes significance based on a t test.
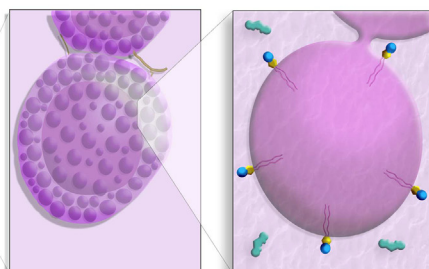
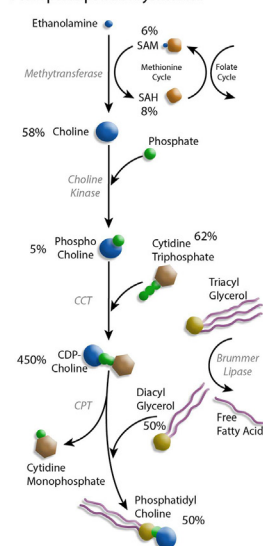## Lipid Metabolism Dysfunction in Aposymbiotic Tsetse Flies

Figure 5. Summary of the dynamics between symbiont loss and reproduction during tsetse fly pregnancy in relation to altered lipolysis

The percentage annotations in the panel depicting the Kennedy Pathway represent the relative amount of the specific lipid metabolite in the aposymbiotic tsetse fly relative to the untreated, wild-type control which would be equivalent to 100%.

transfer of substantial nutritional resources from mother to offspring. A key mechanism in this process is the production of PC by the Kennedy biosynthesis pathway. The *cct1* gene encodes a rate-limiting enzyme in this pathway that prepares the choline head group for attachment to cytidyl triphosphate to generate CDP-choline. CDP-choline is then combined with DAG to produce the final PC product. The observed increase in *cct1* transcripts during pregnancy suggests that this enzyme/pathway is critical to the provision of PC for LD production/function for lipolysis in the FB and for incorporation into products secreted from the MG.[5,32] Production of PC is required to coat the outer surface of LDs produced by the endoplasmic reticulum and is thus critical for lipid metabolism.[36] The amphipathic nature of PC prevents aberrant LD fusion and provides the biochemical conditions to recruit LD-associated proteins required for lipolysis.[37]

In mammals, disruption of appropriate PC-to-phosphatidylethanolamine (PE) ratios in LDs causes the formation of large droplets with reduced surface area. The result is abnormal fat retention within a cell or tissue/organ (steatosis[38]). In tsetse flies, symbiont clearance produces obesity similar to that observed in mammals. Flies initially fed blood spiked with tetracycline are cleared of their *Wigglesworthia*, and any lingering bacterial-derived factors are cleared over subsequent feedings. Symbiont absence results in PC deficiency that inhibits metabolism, mobilization, and integration of stored lipids during lactation. Reproduction is terminated due to lipid retention in the FB and inefficient lipid transfer to the intrauterine larva. This is likely due to the mother's inability to process FB lipids due to dysfunction in LD formation in the FB and MG as *cct1* is abundantly expressed in both locations.

The biochemical observations associated with this phenotype suggest at least two points of failure in PC biosynthesis via the Kennedy (CDP-choline) pathway. The first limiting factor appears to be deficiencies in choline and choline phosphate levels, which form the PC head group. Choline production depends on methylation reactions driven by enzymes that utilize S-adenosyl methionine (SAM) as a cofactor. Prior work in aposymbiotic *Glossina* demonstrates a severe deficiency of SAM in aposymbiotic flies.[15] SAM production depends on the cysteine-methionine pathway, which is dependent on the B vitamins pyridoxal and folate as enzymatic cofactors.[39,40] Methylation reactions require SAM as a cofactor for the production of choline and conversion of PE to PC.[28,41,42] Following the observations in mammalian systems, the mechanistic disruption occurring in the aposymbiotic flies is most likely due to the deficiency in symbiont-derived B vitamins.

Significant disruptions in creatine/creatinine levels were also observed in aposymbiotic flies. These compounds are key regulators of lipid homeostasis for adrenergic and diet-induced thermogenesis in brown and beige adipose tissue in vertebrates. Induced deficiencies in creatine/creatinine in mice result in diet-induced obesity, which can be rescued by dietary creatine supplementation.[43] A similar mechanism could be occurring directly in *Glossina* or through side effects due to deficiencies in other metabolites. The mechanism behind how these compounds regulate lipolysis remains to be elucidated but may also be involved in maintaining lipid homeostasis throughout the reproductive cycle.

Wigglesworthia produces an array of B vitamins (B1, B2, B3, B5, B6, B7, and B9).[14,15,28,35] Deficiencies in B6, folate, and, consequently, SAM have a negative impact on PL biosynthesis in mammalian systems. Issues related to the production of PC have been linked to folate restriction.[44] The aposymbiotic tsetse phenotype resembles the pathology observed in non-alcoholic fatty liver disease (NAFLD) in mammals as PC deficiency is a key dysfunction.[45–47] Rats exhibiting an NAFLD phenotype have been rescued by dietary supplementation with PC.[48] The phenotype observed in aposymbiotic flies (this study and in prior studies[15,28]) is phenotypically equivalent to that observed when *cct1* is suppressed. Folate deficiency is likely a major underlying factor for the phenotype of dysfunctional metabolism of lipids we observe in flies lacking symbionts. Induction of folate deficiency in tsetse through treatment with glyphosate impacts vector competency and reproduction,[28,35] highlighting that folate provision by *Wigglesworthia* represents a critical lynchpin in tsetse metabolism. While female tsetse can survive B vitamin deficiency, the metabolic deficiencies prevent them from mass mobilizing lipids from FB stores to the MG required for milk production during pregnancy.

Another disruption in PC biosynthesis appears to derive from DAG deficiency. The lack of DAGs results in inadequate substrate availability for fusion with cytidine 5′-diphosphocholine by DAG cholinephosphotransferase. DAGs can be synthesized *de novo* during the production of triglycerides (TAGs), or they can be produced during the lipolysis of TAGS upon removal of a fatty acid side chain by a lipase. TAGs are overabundant in aposymbiotic flies, suggesting that lipolysis of the existing triglycerides is compromised under these conditions. In insects, the lipase responsible for this activity is called *brummer lipase* (*Drosophila*, CG5295) and is orthologous to the adipose triglyceride lipase (ATGL) in mammals.[49,50] Brummer and ATGL are both regulated by the phosphorylation state of other LD-bound proteins, called perilipins.[51] Perilipins sequester a co-activator of ATGL in their non-phosphorylated state called CGI-58, which inhibits lipolysis.[52] Recent work demonstrates that LDs lacking proper PC levels show increased levels of associated perilipins and steatosis (lipid accumulation) phenotype.[38] The PC deficiencies in aposymbiotic *Glossina* could be functioning via the same paradigm, resulting in perilipin accumulation and inhibition of *brummer*-mediated lipolysis resulting in DAG deficiency and TAG accumulation.

This study demonstrates that lipid metabolism, a foundational process in *Glossina* biology, is impaired without their obligate symbionts. This is likely a model for other symbiotic systems, particularly those compensating for dietary nutritional deficiencies, such as blood-feeding invertebrates that are supplied with B vitamins from bacterial symbionts. This includes ticks,[53] bed bugs,[54] and lice,[23] which are all epidemiologically important blood-feeding disease vectors and require obligate symbionts to provide B vitamins. Beyond blood-feeding arthropods, specific bacteria associated with plant-feeding insects also provide B vitamins,[55] suggesting that lipid metabolism and B vitamin dependency issues likely occur in many systems. Lastly, B vitamins can be provided to insect species with transient bacterial symbionts, such as mosquitoes,[56] suggesting that the interdependence of B vitamins and lipid metabolism may be a widespread phenomenon among arthropod species. As a key example, mosquitoes display altered lipid metabolism related to microbiome elimination and B vitamin deficiency.[56–59]

Along with the role among arthropod systems, genomic studies have identified significant functional convergence among live-bearing systems in invertebrate and vertebrate lineages.[27] This includes aspects that range from metabolism to immune function.[27] In the vertebrate systems, fetal development requires essential fatty acids and long-chain polyunsaturated fatty acids.[60–62] As in the insect system, the accumulation of lipids early in vertebrate pregnancy is a critical feature that builds up stores providing lipids to the developing progeny.[60] As pregnancy progresses, the mother transitions from lipogenesis to lipolysis to increase the lipid supply to the fetus.[63,64] An imbalance in lipid levels provided to the neonates, either through malnutrition of the mother or impaired transfers, can yield many negative consequences ranging from delayed growth/development to death.[60,65] Similar to this study, B vitamin levels have been

documented to alter lipid metabolism in mammalian systems, focusing mostly on human and murine systems.[60,66,67] Low B vitamin levels during pregnancy have been directly associated with a reduction in circulating lipid levels, increased obesity in the mother, and a reduction in the health of the neonate,[66,68,69] which is functionally similar to the obesity and reduced larval growth we observed in tsetse flies lacking B vitamins produced by *Wigglesworthia*. As observed in this study for tsetse flies, the induction of obesity acts through impaired PC synthesis that requires specific B vitamins, which is also critical for vertebrate lipid metabolism.[70] Thus, a distinct commonality of viviparity among animals is the requirement of specific B vitamins for lipid metabolism to meet the energetic demand of internal progeny development.

Limitations of the study

Our results reveal that removal of symbiotic bacteria from tsetse flies impacts PC biosynthesis, which in turn causes obesity in the mother and induces abortion. Studies on the removal of symbionts in tsetse flies require antibiotic treatment, which could have other unintended consequences. This has been a general limitation in studies on the interactions between tsetse flies and *Wigglesworthia*. This has prevented studies where aposymbiotic tsetse flies can be monocolonized with *Wigglesworthia* to allow for the recovery of a control phenotype. Tsetse flies do harbor only a single, consistent symbiont (*Wigglesworthia*), with other species showing very little impact on tsetse biology or found very rarely in the population,[19–21] suggesting that phenotypes observed are most likely from *Wigglesworthia*. Thus, until *Wigglesworthia* can be cultured (has yet to be accomplished) and monocolonized back into tsetse flies (has yet to be accomplished), the role of other factors in these phenotypes cannot be fully excluded.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107108.

AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit α-GmmMGP antibodies	Attardo et al.[71] Attardo et al.[72]	NA
α-Digoxigenin-rhodamine	Roche Applied Science	11207750910
Alexa Fluor 488 goat α-rabbit IgG	Invitrogen (Sigma-Aldrich)	A-11008
Bacterial and virus strains		
pGEM T-Easy vector	Promega	PRA1360
Chemicals, peptides, and recombinant proteins		
TRizol reagent	Invitrogen	15596018
Folic acid	Sigma-Aldrich	F8758
Vanillin reagent	Thermo Scientific	A1116922
Glyphosate	Sigma	45221
VECTASHIELD Mounting Medium	Vector laboratories	H-1700-2
Nile Blue stain	Sigma-Aldrich	N0766
Isopropanol	Fisher Scientific	BP26181
Ethanol	Fisher Scientific	BP28184
Yeast extract	Fisher Scientific	BP1422
Bovine blood	Lampire Biologicals	7230801
Tetracycline	Sigma-Aldrich	T3258
Chloroform	Fisher	C607
Critical commercial assays		
TURBO DNA Free kit	Invitrogen	AM1907
RNeasy Mini kit	Qiagen	74104
Superscript III reverse transcriptase	Invitrogen	18080093
QIAquick PCR purification kit	Qiagen	28104
Block-iT Dicer RNAi kit	Invitrogen	K3600-01
MAXIscript T7 transcription kit	Invitrogen	AM1314
Phosphatidylcholine assay	Caymen Chemicals	10009926
Vitamin B6 enzymatic assay	Sigma-Aldrich	MAK425
MEGAscript RNAi Kit	Ambion	AM1626
Deposited data		
Metabolomics data	This paper	https://data.mendeley.com/datasets/7wg7kv23jf/1
Experimental models: Organisms/strains		
Glossina morsitans morsitans	Yale strain	NA
Oligonucleotides		
Customized oligonucleotides for RT-PCR and qRT-PCR	Integrated DNA Technologies	N/A
Customized oligonucleotides for dsRNAi synthesis	Integrated DNA Technologies	N/A
See Table S1 for list of primers	N/A	N/A
Software and algorithms		
Adobe Photoshop and Illustrator	Adobe	https://www.adobe.com/
Infinity1 USB 2.0 camera and software	Lumenera Corporation	www.lumenera.com

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Zeiss Zen imaging software	Zeiss	www.zeiss.com
RStudio, vegan package	Oksanen et al. 2022[73]	https://cran.r-project.org/web/packages/vegan/index.html
Metaboanalyst software package	Metaboanalyst	https://www.metaboanalyst.ca/
CFX manager software version 3.1	Bio-Rad	www.bio-rad.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Joshua B. Benoit (joshua.benoit@uc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

● Data: Data is available at the https://data.mendeley.com/datasets/7wg7kv23jf/1

● Code: This paper does not report original code.

● Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Flies

Glossina morsitans morsitans flies were maintained on defibrinated bovine blood (Lampire Biologicals, Pipersville, PA, USA) at 25°C and 50–60% RH using an artificial membrane feeding system[74] at the insectaries at Yale School of Public Health and the Slovak Academy of Sciences. Females were mated 3–5 days after emergence. Flies were collected according to established developmental markers based on oocyte, embryo and larva presence.[71,75,76] Aposymbiotic tsetse larvae were derived from females fed a diet supplemented with tetracycline (20 μg per mL of blood) to clear their indigenous microbiota, predominantly *Wigglesworthia*.

METHOD DETAILS

Milk extraction

Milk products were extracted from the guts of developing third instar larvae based on our previous studies.[7,77] Briefly, actively-feeding individuals were removed from the uterus and their digestive tracts were dissected. A pulled glass capillary needle was utilized to pierce into the digestive tract and the contents were removed using reverse pressure. Each sample was stored at −80°C until utilization in the experiments.

Lipidomics

Tissues (milk glands and fat body) were dissected from aged-matched WT flies that received normal blood meals for five weeks (control - symbiotic) and aposymbiotic females and prepared according to our previously established protocol.[15] Briefly, tissues were added 100 μL of ice-cold 1X phosphate buffered saline (PBS), homogenized with a pestle and stored at −80°C. For both aposymbiotic and control flies, samples consisted of four replicates of 20 individuals each. Samples were shipped to Metabolon (Morrisville, NC, USA) and analysis was performed using the proprietary Metabolon DiscoveryHD4 global lipidomics platform. Each sample was screened for the presence and quantity of lipids by mass spectrometry using a panel of specific lipids. Relative differences between samples were determined by comparison of mean metabolite abundance across the biological replicates. Significance of the difference between symbiotic and aposymbiotic samples was determined by Welch's two-sample t-test: p value cut off of (<0.05). False discovery rate (FDR) was tested for by q-value with a cut off of (<0.1). Non-Metric Multidimensional Scaling

(NMDS) plots were created using the metaMDS function from the R package vegan[78,79] to determine differences in the general lipid composition between control and aposymbiotic individuals. Analysis of similarities (ANOSIM, using the anosim from the R package vegan) tests was performed to test for significance in the general differences.

RNA extraction and qPCR

RNA was extracted from flies with TRizol reagent (Invitrogen, Carlsbad, CA, USA) using the manufacturer's recommended protocol. RNA was treated with TURBO DNA Free kit (Ambion, Austin, TX, USA). RNA was precipitated with alcohol to remove residual salt, purified with a RNeasy kit (Qiagen, Maryland, USA) and stored at −70°C until use. cDNA was prepared using the Superscript III reverse transcriptase (Invitrogen).

Transcript abundance was determined utilizing quantitative PCR (qPCR) on a Bio-Rad CFX detection system (Hercules). Primer sequences used were those from Table S1. Results were analyzed with CFX manager software version 3.1 (Bio-Rad). Ct values for genes of interest were standardized by Ct values for the control gene (tubulin) and relative to the average value for the control treatment or newly emerged flies, yielding a relative fold change in gene expression.

RNA interference of *cct1*

Suppression of *cct1* was performed utilizing double-stranded RNA (dsRNA) according to our previous studies on tsetse gene suppression.[17,77] The T7 promoter sequence was added to the 5′ end of the primer sequences and PCR amplification conditions are described (Table S1). The PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into pGEM T-Easy vector (Promega, Madison, WI) and verified by sequencing (Keck DNA sequencing facility, Yale University). dsRNAs were synthesized using the MEGAscript RNAi Kit (Ambion, Austin, TX), purified using a RNeasy Mini Kit (Qiagen, Valencia, CA) and siRNAs were generated by using the Block-iT Dicer RNAi kit (Invitrogen, Carlsbad, CA). The siRNA concentration was adjusted to 600–800 ng/μL in PBS and each fly was injected with 1.5 μL siRNA using a pulled glass capillary needle into their thorax. Concentration of siRNAs were kept at 600–800 ng/μL within the 1.5 μL injected. Knockdown efficiency was determined 5days after injection by qPCR and normalized to *tubulin* levels. Fecundity following *cct1* was assessed according to previously established protocols.[7,77]

Glyphosate treatment

Groups of *G. morsitans* flies were maintained on a diet containing 100 μM glyphosate combined with 500 nM folic acid [N-(phosphonomethyl)glycine; Sigma-Aldrich, St. Louis, MO, USA] or (Sigma-Aldrich). This treatment has been previously shown to impact folate synthesis.[28,35] Following six bloodmeals, the flies were examined to determine if lipid and phosphatidylcholine levels were increased or decreased. These treatments were conducted to eliminate potential off-target effects of tetracycline supplementation to validate *Wigglesworthia*'s role in tsetse lipid metabolism, and to provide a secondary validation of impaired lipid metabolism without treatment with antibiotics.

Total lipid assay

Total lipids present within biological samples were determined with a standard vanillin assay.[9,80,81] Whole flies and individual tissues were dried at 0% RH at 60°C and weighed to determine the dry mass. The flies were homogenized in 2 mL of chloroform:methanol (2:1). The supernatant was removed and placed into a glass tube and the solvent was evaporated at 90°C. The dried lipids were treated with 0.4 mL of concentrated sulfuric acid at 90°C for 10 min. The acid/lipid mixture (40 μL) was combined with 4 mL vanillin reagent. Samples were measured spectrophotometrically at 525 nm and the total lipid content was calculated against a lipid standard.

In situ hybridization for *cct1*

Milk gland tubules and fat body were removed from tsetse fly females at six-seven days after the previous birth. The combined milk gland/fat body was placed into Carnoy's fixative for a five-six day fixation period.[4,72] Antisense/sense digoxigenin-labeled RNA probes for *cct1* were generated using the MAXIscript T7 transcription kit following manufacturer's protocol (Ambion, Austin, TX, USA) using a primer set with a T7 primer (Table S1). Antibody solutions were made using α-Digoxigenin-rhodamine, Fab fragments (Roche Applied Science, Penzberg, Germany) for FISH probe detection (1:200 dilution) and rabbit

α-GmmMGP (1:2500) antibodies. Alexa Fluor 488 goat α-rabbit IgG (Invitrogen) at a dilution of 1:500 was added as a secondary antibody for immunohistochemistry.[4,72] Slides were mounted in VECTASHIELD Mounting Medium with DAPI (Vector laboratories Inc. Burlingame, CA, USA). Samples were observed using a Zeiss Axioskop2 microscope (Zeiss, Thornwood, NY, USA) equipped with a fluorescent filter and viewed and imaged at 400× magnification. Images were captured using an Infinity1 USB 2.0 camera and software (Lumenera Corporation, Ottawa, ON, Canada) and merged in Adobe Photoshop.

Phosphatidylcholine assay

Phosphatidylcholine levels were assessed through the use of a colorimetric assay (Caymen Chemicals, Ann Arbor, MI, USA). Extracted milk from the guts of third instar larvae (10 μL) was placed within the sample well of a 96 well plate. The colorimetric reaction was initiated by adding 100 μL of the reaction mixture. Following mixing for a few seconds, the plate was incubated at 37°C for 60 min and the concentration was determined by comparison to a phosphatidylcholine standard.

Vitamin B6 assay

Vitamin B6 levels were quantified based on previous studies in *Glossina*.[16] A Sigma-Aldrich vitamin B6 enzymatic assay, with minor modifications to the manufacturer's protocol, was used to examine the levels of pyridoxal 5′-phosphate (Vitamin B6). Specifically, all reaction mixtures were scaled down 5 times and assembled in 0.6-mL Eppendorf tubes. Absorbance (optical density [OD] at 546 nm) was measured using a Biotek Synergy H1 plate reader. Vitamin B6 concentrations were determined via comparison to a standard curve made from calibrators provided with the assay kit.

Nile Blue staining

Lipid composition of fat body cells from control and aposymbiotic *Glossina* was analyzed microscopically using the lipophilic Nile Blue stain (Sigma). Nile Blue is a differential stain, which results in charged phospholipids (fatty acids, chromolipids and phospholipids) being stained blue and neutral lipids (triglycerides, cholesterol esters, steroids) being stained red/pink.[82] The method used here is a modified version of the protocol described by Canete and colleagues.[83] Fat body tissues were dissected from flies reared as described in the Lipidomics section above. Dissected tissues were immediately transferred to ice-cold 4% paraformaldehyde in PBS and allowed to fix overnight at 4°C. Fixed tissues were then rinsed in PBS followed by immersion in 0.1 mg/mL aqueous Nile Blue sulfate (Sigma-Aldrich) for 30 min. After staining, tissues were rinsed three times with PBS for 1 min per rinse to remove excess stain. Tissues were then mounted in glycerol on single depression concave microscope slides. Stained fat bodies were visualized using a Zeiss Axio Vert.A1 scope and images captured using the Zeiss Zen imaging software. Color differences were quantified by measuring the average amount of red and blue color in five 100 pixel squares within the center of each lipid droplet with the use of Adobe Photoshop.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses

Lipidomics data were analyzed using the Metaboanalyst software package (https://www.metaboanalyst.ca/).[84] Metabolite abundance values represent peak area counts measured from compound peaks. Samples were normalized across samples using the sum of raw area counts for each sample. Missing values were imputed by replacement with minimum detected values for each metabolite. Data were rescaled by median centering to a value of 1 for each sample. Significant differences between control and aposymbiotic samples were measured by t-test followed by p value correction using false discovery rate (FDR)/ Benjamini and Hochberg analysis. Differences in relative abundance were considered significant below an FDR corrected P-value of 0.05. Differences in other biological attributes were assessed through the use of t-test or ANOVA through the use of R packages.