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Vitamin B_6 Generated by Obligate Symbionts Is Critical for Maintaining Proline Homeostasis and Fecundity in Tsetse Flies

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The viviparous tsetse fly utilizes proline as a hemolymph-borne energy source. In tsetse, biosynthesis of proline from alanine involves the enzyme alanine-glyoxylate aminotransferase (AGAT), which requires pyridoxal phosphate (vitamin B_6) as a cofactor. This vitamin can be synthesized by tsetse's obligate symbiont, *Wigglesworthia glossinidia*. In this study, we examined the role of *Wigglesworthia*-produced vitamin B_6 for maintenance of proline homeostasis, specifically during the energetically expensive lactation period of the tsetse's reproductive cycle. We found that expression of *agat*, as well as genes involved in vitamin B_6 metabolism in both host and symbiont, increases in lactating flies. Removal of symbionts via antibiotic treatment of flies (aposymbiotic) led to hypoprolinemia, reduced levels of vitamin B_6 in lactating females, and decreased fecundity. Proline homeostasis and fecundity recovered partially when aposymbiotic tsetse were fed a diet supplemented with either yeast or *Wigglesworthia* extracts. RNA interference-mediated knockdown of *agat* in wild-type flies reduced hemolymph proline levels to that of aposymbiotic females. Aposymbiotic flies treated with *agat* short interfering RNA (siRNA) remained hypoprolinemic even upon dietary supplementation with microbial extracts or B vitamins. Flies infected with parasitic African trypanosomes display lower hemolymph proline levels, suggesting that the reduced fecundity observed in parasitized flies could result from parasite interference with proline homeostasis. This interference could be manifested by competition between tsetse and trypanosomes for vitamins, proline, or other factors involved in their synthesis. Collectively, these results indicate that the presence of *Wigglesworthia* in tsetse is critical for the maintenance of proline homeostasis through vitamin B_6 production.

Tsetse flies (*Glossina* spp.), which are distributed throughout sub-Saharan Africa, are vectors of human and animal African trypanosomiasis. Several aspects of the tsetse's physiology are distinctive from those of other insects, including viviparous reproduction, exclusive reliance on proline as the major energy source, and dependence on obligate symbionts for fecundity (1–5). Due to their viviparous reproductive physiology, tsetse females are limited to a maximum of 8 to 12 progeny during their lifetime (1). During each gonotrophic cycle, a single offspring undergoes embryonic and complete larval development within the female's uterus (expanded reproductive tract) (6). Immediately after birth, deposited larvae burrow into the ground, pupate, and remain dormant until eclosion (6). All nutrients required for larval and pupal development are provisioned through the female accessory gland (milk gland) in the form of intrauterine milk secretions (1, 7, 8). During each lactation cycle, the milk gland secretes up to 25 mg of milk that consists primarily of lipids and proteins (9). Twelve proteins have been identified as the major constituents of tsetse milk (10–13). Lipids present in tsetse milk are generated either from metabolized blood meals or via lipolysis of stored fat body lipids (14–17). During lactation, the rapid incorporation of nutrients into the milk reduces total maternal lipid content by nearly 50% and protein content by about 25% (14, 18, 19). The nutrients are transported to the milk gland as free amino acids, free fatty acids, and lipophorin-associated diacylglycerol (15, 18, 19). We recently showed that this process is regulated by interactions between juvenile hormone and insulin-like peptides, where high levels of both promote increased lipogenesis between bouts of lactation while low levels facilitate the increased lipid breakdown observed during milk production (20).

The tsetse relies on proline as a precursor to generate ATP via the tricarboxylic acid (TCA) cycle. This process differs from that of most other insects, which utilize a form of carbohydrate, such as glucose or trehalose, as a substrate for the TCA cycle (21, 22). The tsetse is one of a few insects that rely on free amino acids as a circulating resource to be used in the TCA cycle (21, 22). Under this circumstance, proline is utilized to produce glutamate and then oxaloacetate for inclusion in the TCA cycle. This process generates alanine as a final product, which is transported back to the fat body for proline regeneration (21–23). The metabolic machinery necessary to convert proline into alanine and to regenerate proline from alanine is generally conserved among insects. Analysis of the tsetse genome did not identify any specific gene expansions associated with proline metabolism (23). The first step in the conversion of alanine to proline in the fat body is mediated by the enzyme alanine-glyoxylate aminotransferase (AGAT) (23), which employs the cofactor pyridoxal phosphate (the active form of vitamin B_6) (Fig. 1). The regeneration of proline also requires acetyl coenzyme A (acetyl-CoA), which is generated from the β-oxidation of fatty acids that are provided by the breakdown of fat body-derived triglycerides (21, 24). Comparative analysis of

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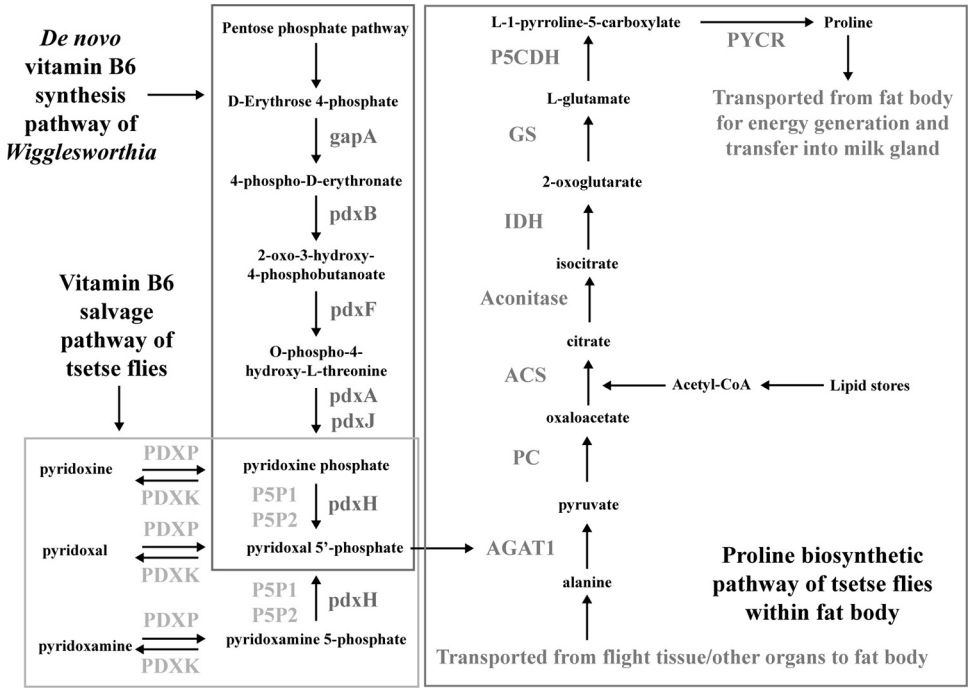

FIG 1 Interactions of synthesis of vitamin B_6 by *Wigglesworthia* with salvage of vitamin B_6 and proline synthesis in tsetse flies. Enzymes in metabolic pathways: pyridoxal phosphatase (PDXP), pyridoxal kinase (PDXK), pyridoxamine 5′-phosphate oxidase 1 (P5P1), pyridoxamine 5′-phosphate oxidase 2 (P5P2), glyceraldehyde 3-P dehydrogenase A (gapA), erythronate-4-phosphate dehydrogenase (pdxB), phosphoserine aminotransferase (pdxF), 4-hydroxythreonine-4-phosphate dehydrogenase (pdxA), pyridoxal phosphate biosynthetic protein (pdxJ), pyridoxine-phosphate oxidase (pdxH), alanine-glyoxylate transaminase (AGAT), pyruvate carboxylase (PC), ATP citrate synthase (ACS), aconitate hydratase (aconitase), isocitrate dehydrogenase (IDH), glutamate synthase (GS), 1-pyrroline-5-carboxylate dehydrogenase (P5CDH), pyrroline-5-carboxylate (PYCR). Pathways were established based on sequences from the *Wigglesworthia* (2, 32) and *Glossina* (41) genomes.

transcriptomes from pregnant females and females immediately after birth indicated that *agat* is one of the few upregulated genes in lactating flies (12). This finding suggests that AGAT may play a critical role in tsetse fecundity by facilitating development of intrauterine progeny through the maintenance of proline homeostasis.

Tsetse harbor two symbiotic bacteria that colonize developing larvae via maternal milk secretions (25, 26). One of these microbes, obligate *Wigglesworthia glossinidia*, is required for the fertility of adult females. Tsetse's second milk-associated bacterial symbiont, the commensal *Sodalis glossinidius*, lacks a well-defined functional association with its host (25–27). In natural populations, all tsetse harbor *Wigglesworthia* bacteria while *Sodalis* prevalence varies between different species and populations (28–31). The putative proteomes of both symbionts indicate that they have the ability to synthesize biotin (vitamin B_7), thiazole (component of vitamin B_1), lipoic acid, riboflavin (vitamin B_2), folate (vitamin B_9), pantothenate (vitamin B_5), pyridoxine (vitamin B_6), protoheme, and nicotinamide (vitamin B_3) (2, 30). However, only *Wigglesworthia* is capable of generating thiamine (vitamin B_1) (2, 30, 31). Interestingly, *Sodalis* scavenges *Wigglesworthia*-generated thiamine via a functional thiamine ABC transporter (32, 50). Removal of *Wigglesworthia* via administration of antibiotics to mated females disrupts the tsetse's reproductive processes (4, 33). This phenotype can be partially rescued, through an unknown mechanism, by supplementation of the diet of pregnant females with a cocktail of either B vitamins or yeast extract (3, 5).

The goal of this work is to understand the function that *Wigglesworthia* plays in tsetse physiology with a focus on the synthesis of B vitamins (specifically vitamin B_6), maintenance of proline homeostasis, and impact on reproduction. We describe the role of a vitamin B_6-dependent enzyme (AGAT) in the proline synthesis pathway relative to the presence of B vitamins produced by *Wigglesworthia*. As parasitic trypanosomes also utilize proline as an energy source during their development in the tsetse host, we tested the effect of trypanosome infection on host proline levels. Our study indicates that *Wigglesworthia* is critical to vitamin B_6 supplementation in order to maintain proline homeostasis through reproduction and during trypanosome infection.

MATERIALS AND METHODS

Biological materials. The tsetse fly (*Glossina morsitans morsitans*) colony used for the experiments originated from a population of flies in Zimbabwe collected in the mid-1970s. Flies are maintained in the Yale University Insectary at 24°C with 50 to 55% relative humidity on defibrinated bovine blood provided every 48 h via an artificial feeding system (34). The colony has been supplemented with puparia obtained from other *G. morsitans morsitans* colonies on multiple occasions since 1990. Symbiont-cured (aposymbiotic) lines were developed according to the methods of Pais et al. (3) and Weiss et al. (35). Briefly, wild-type (WT) females received blood meals supplemented with 40 μg/ml tetracycline and yeast extract (1%). Symbiont-free progeny from antibiotic-treated females were collected, and subsequent adults were designated Apo (35). The absence of *Wigglesworthia* in adult Apo flies was confirmed by microscopic examination of the midgut bacteriome organ (*Wigglesworthia*-containing organ) and by quantitative PCR (qPCR) of two *Wigglesworthia*-specific genes (*pdxB* and *rpsC*) (see Fig. S1 in the supplemental material).

TABLE 1 B vitamin cocktail utilized to supplement blood

B vitamin in mixture	Concn (μg/ml)
Choline chloride	185
Mesoinositol	118
Thiamine (B_1)	100
Nicotinic acid (B_3)	100
Pantothenic acid (B_5)	100
Pyridoxine (B_6)	100
Folic acid (B_9)	30
Riboflavin (B_2)	20
Cobalamin (B_{12})	1
D-Biotin (B_7)	1

Dietary supplementation experiments for fecundity recovery analysis. Dietary supplementation experiments were conducted to identify the factors that recover fecundity and proline levels in Apo flies. For all experiments, females were mated at 5 days posteclosion. Apo flies were provided with 1% (mass/vol) yeast extract at each blood meal according to the work of Pais et al. (3). These flies were designated Apo+Y flies. *Wigglesworthia*-containing bacteriome extracts were obtained by dissecting five bacteriomes from WT females and homogenizing them in 100 μl phosphate-buffered saline (PBS) as previously described (36). *Wigglesworthia* extract was added to 2 ml of blood provided to Apo females three times per week. These flies are designated Apo+Wig flies. Lastly, we supplemented Apo females with a diet consisting of a variety of B vitamin cocktails. The concentration of B vitamins was based on a modification of the protocol used for other obligate blood-feeding insects (37, 38) that leads to a recovery of negative phenotypes in the absence of symbionts (Table 1). With this vitamin cocktail, we generated three treatment lines for Apo flies: full vitamin cocktail (Apo+VitB flies), full vitamin cocktail minus vitamin B_6 (Apo+VitB−B6 flies), and vitamin B_6 alone (Apo+VitB6 flies). The Apo flies were provided these vitamins at each blood meal. All flies were provided three blood meals per week throughout the course of their lifetime.

Following supplementation with microbial extracts and vitamins, multiple physiological characteristics were measured in female flies. Proline levels were measured throughout development in WT and Apo females. Vitamin B_6 levels were assessed in mated flies immediately before lactation (16 days posteclosion) for WT and Apo females with and without each type of dietary supplementation. Fecundity was assessed as total larval deposition number for groups of WT and Apo females with and without each type of supplementation over two bouts of pregnancy. In addition, proline levels and fecundity were assessed for Apo females subjected to *agat1* short interfering RNA (siRNA) treatments as described below.

RNA isolation and quantitative PCR (qPCR). TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to recover total RNA from flies according to the recommended protocol. RNA was subsequently treated twice with the Turbo DNA Free kit (Ambion, Austin, TX, USA) to remove DNA, cleaned with an RNeasy kit (Qiagen, Maryland, USA), and held at −70 C until use. cDNA was prepared using a Superscript III reverse transcriptase kit (Invitrogen). PCR was performed with a gene-specific primer pair (Table 2) with the GoTaq DNA polymerase kit (Promega). The PCR amplification conditions were as follows: 95°C for 3 min, 30 to 40 cycles of 30 s at 95°C and 52 to 60°C for 1 min, and 1 min at 70°C using a Bio-Rad DNA Engine Peltier thermocycler (Hercules, CA).

Transcript abundance was determined by qPCR analyses on a CFX PCR detection system (Bio-Rad, Hercules, CA). Results were analyzed with CFX Manager software version 3.1 (Bio-Rad). Primer sequences used were those from Table 2. Threshold cycle (C_T) values for genes of interest were standardized by C_T values for the control genes (*tubulin* for tsetse and the ribosomal protein gene *rpsC* for *Wigglesworthia*) and relative to the average value for the control treatment or newly emerged flies, yielding the delta C_T value.

Vitamin B_6 measurement. Vitamin B_6 levels were quantified in bacteriomes and carcasses ($n = 5$ per tissue) harvested from wild-type and aposymbiotic females 15 days postmating. This procedure was performed using a Bühlmann vitamin B_6 enzymatic assay, with minor modifications to the manufacturer's (Bühlmann Laboratories) protocol. 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 Nanodrop 2000 spectrophotometer (Thermo Scientific), and vitamin B_6 concentrations in tsetse tissues were determined via comparison to a standard curve made from calibrators provided with the assay kit.

Proline measurements. Free proline levels were determined according to the method of Bergman and Loxley (39) with the modification by Misener et al. (40). For whole-body analyses, female flies were dried, weighed, and homogenized in 600 μl PBS. Following homogenization, the sample was centrifuged at 5,000 rpm and 100 μl was obtained for the assay. Hemolymph (2 μl/fly) was acquired from flies utilizing reverse pressure through a pulled glass capillary needle inserted into the thorax. Glacial acetic acid (300 μl) and ninhydrin solution (300 μl; 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) were

TABLE 2 Quantitative PCR primers and siRNA sequences

Primer type and name	Sequence	
	Forward	Reverse
qPCR		
agat1	AAAAGTGAAACGAGCCTTAG	ATTTCGCAGTTAATTTCTCC
agat2	TCTTCATTGGCATAGAATTG	AGAATTTTGTGCATTTCCTT
agat3	AGGCTTGAAAGCATTTATTC	AGTCTTTCATTGGCATTCTT
pdxB	GCGGTGCTGTTGTAGATAAT	GACAACAAGTCCAAGCAAAT
pdxH	CAAATTTTATTTTTAGGTACTGCAA	GCCCAGATGCTAATTTGACT
pdxJ	GAAATGGCTCCTACAGAAAA	ATGATATCAAGACCGCTTTC
pdxK	CAGTTTTGGGTGATAATGGT	GCTTCATATTGATTGGGTGT
gapA	CAACAAATTGCTTAGCTCCT	ATCTACAGGATTTTGCGTTG
p5p1	TATTGAAGGTAAAGCGGAAA	ATGCGAGTACCTTGCTCACT
p5p2	GGCGACAGTCAATAGTAAGG	CGATATCGTTAGCTTTACCG
phospho2	GTTTTCCGTTTATTGCATTC	TTGTGATTTTGTCTCAAACG
siRNA		
agat1 (set 1)	UUCAAAUCACCUGGGAAUUUCGCAGUU	AAGUUUAGUGGACCCUUAAAGCGUC
agat1 (set 2)	AACAGCAGCCAAUGGAAAUCCGUUGCC	TTGUCGUCGGUUACCUUUAGGCAAC

added to the sample and held at 100°C for 60 min. The reaction was terminated by placing sample tubes in an ice bath for 5 min. The mixture was combined with 600 μl toluene, vortexed, and incubated at room temperature for 30 min. The chromophore-containing upper phase was removed, and optical density was determined at 520 nm. Proline concentrations of the samples were determined in relation to a reference proline standard curve. Proline levels were assessed at least 24 h post-blood meal to reduce the impact of fluctuations that occur due to digestion on the measured levels (1).

RNAi of *agat1*. RNA interference (RNAi) was accomplished by injection of short interfering RNAs (siRNAs). Two sets of duplex sequences (Table 2) were designed targeting the 5′ and 3′ ends of the alanine-glyoxylate aminotransferase 1 gene (*agat1*) and synthesized by Integrated DNA Technologies (IDT). A bioinformatic homology analysis was conducted on the two siRNA sequences against the predicted genes from the tsetse genome (40) and a *de novo* library generated in a previous study (12) to ensure *agat1*-specific targeting. Control siRNAs homologous to green fluorescent protein (GFP) were used as controls (Table 2). The 5′ and 3′ siRNA oligonucleotides were combined and diluted to a concentration of 1.5 μg/μl. Each mated female was injected with 2 μl of siRNA solution 10 to 12 days after adult emergence. Validation of knockdown was accomplished via qPCR 4 to 5 days after siRNA injection.

Trypanosome infections. Teneral WT adults (48 h posteclosion) received 2×10^6 infective bloodstream-form *Trypanosoma brucei brucei* organisms per 1 ml of blood in their first meal and were subsequently maintained on regular blood meals. A control group was established that received only uninfected blood meals. All females were mated 3 days post-first blood meal acquisition. Forty days after trypanosome challenge, all flies were dissected and their salivary glands were microscopically examined for the presence of parasite infections. Hemolymph (2 μl/fly) was obtained from infected male and female flies as well as from control uninfected flies. Control groups were of the same age, and all females analyzed were carrying the second larval instar at the time of dissection. Proline content within the hemolymph was measured as described above.

Statistics. Changes in gene expression and proline levels were analyzed with one- or two-way analysis of variance (ANOVA) following tests of parametric assumptions along with the Bonferroni correction and Dunnett's test. Where noted, the Student-Newman-Keuls (SNK) comparison was used to test for significances over time. Data not meeting parametric assumptions were log transformed. All data are presented as means ± standard errors (SEs) with statistical significance set at a P of <0.05.

RESULTS

Expression of *agat1* throughout pregnancy. AGAT functions in the first step for conversion of alanine to proline. *In silico* analysis of tsetse genome data identified three genes that encode a family of AGAT proteins (41). qPCR-based expression analysis with gene-specific primers revealed that transcript levels of only one member of the family, *agat1*, varied throughout tsetse pregnancy. The expression of this gene increased during embryogenesis and early lactation, decreasing during late lactation prior to birth, and increased again during lactation in the second gonotrophic cycle (Fig. 2A). Tissue-specific expression analysis of *agat1* showed high levels in the fat body/milk gland and Malpighian tubules relative to other tissues (Fig. 2B). These results suggest that high-level *agat1* expression in the fat body/milk gland organ and Malpighian tubules may be critical during the lactation process in tsetse pregnancy.

Expression of vitamin B_6 metabolism-related genes in tsetse and *Wigglesworthia* throughout pregnancy. To determine the potential interactions between pyridoxal phosphate produced by *Wigglesworthia* and the host vitamin B salvage pathway (2, 41), we measured the expression of five genes (*pdxB*, encoding erythro-

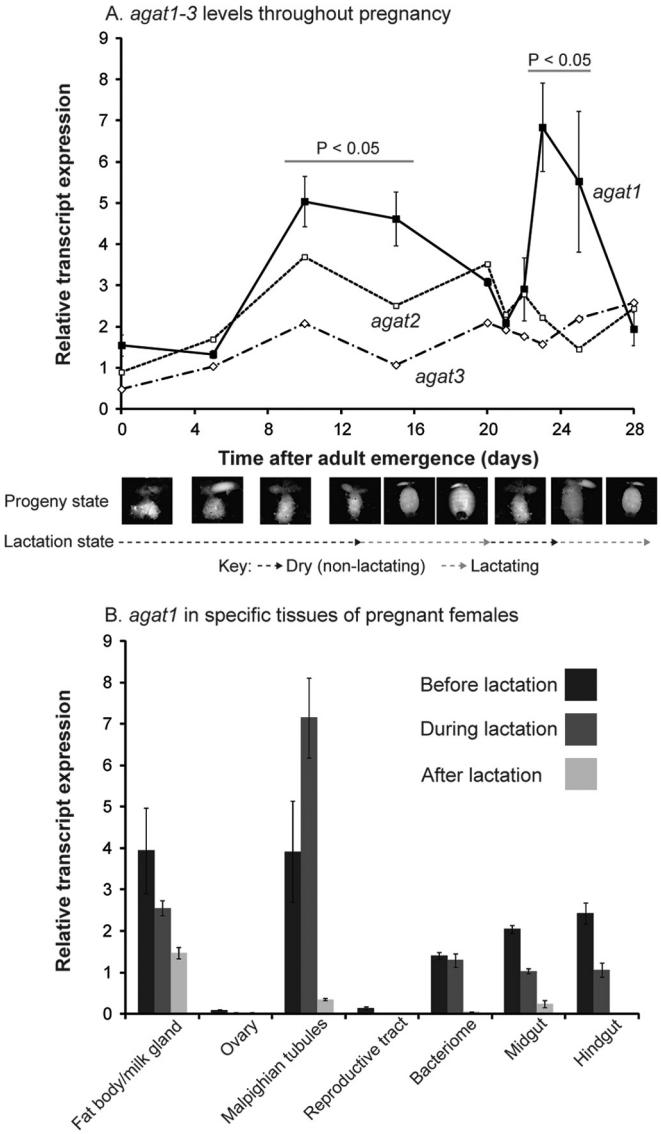

FIG 2 Expression of alanine-glyoxylate transaminase 1 to 3 genes (*agat1* to *agat3*). (A) Temporal expression. (B) Tissue localization. Transcript levels were determined by qPCR analysis. The data were analyzed with CFX Manager software, version 3.1 (Bio-Rad). Data represent the means ± SEs for four samples and were normalized to *tubulin*. Error bars are omitted from *agat2* and *agat3* data (no significant differences among treatments).

nate-4-phosphate dehydrogenase; *pdxH*, encoding pyridoxine phosphate oxidase; *pdxJ*, encoding pyridoxal phosphate biosynthetic protein; *pdxK*, encoding pyridoxal kinase; and *gapA*, encoding glyceraldehyde 3-phosphate dehydrogenase) involved in vitamin B_6 synthesis in *Wigglesworthia* from whole adult mated WT females. We found that *pdxB* and *pdxH* expression increased 6-fold during the lactation period in mated females. Following birth, the expression of this gene decreased 2- to 3-fold (Fig. 3A). Three genes within the tsetse salvage pathway also underwent expression analysis. The three genes (*p5p1*, encoding pyridoxamine 5′-phosphate oxidase 1; *p5p2*, encoding pyridoxamine 5′-phosphate oxidase 2; and *phospho2*, encoding pyridoxal phosphatase) had expression profiles similar to those of *pdxB* and *pdxH* during pregnancy (Fig. 3B). Localization of *pdxB* revealed high transcript

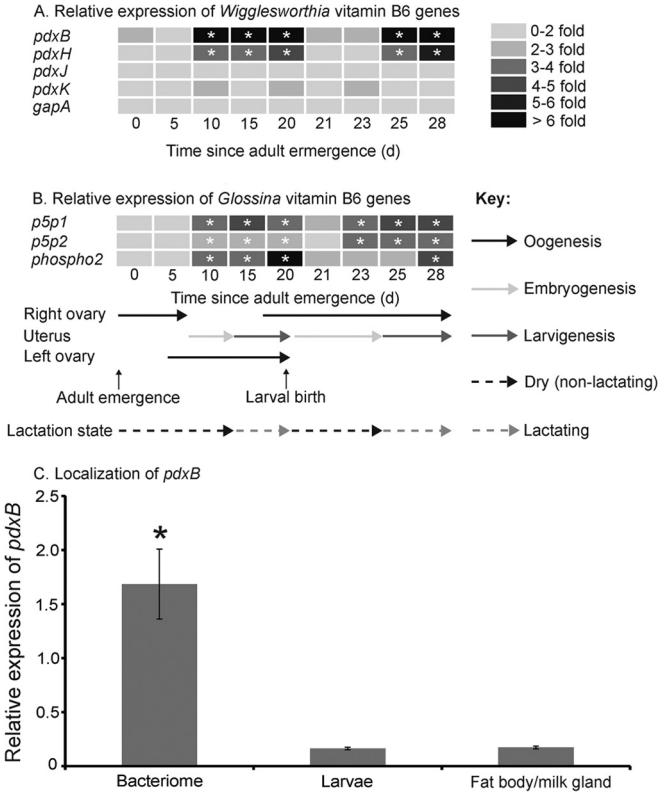

FIG 3 Expression levels of selected genes from *Wigglesworthia* and *Glossina* associated with vitamin B_6 metabolism. (A) Transcript levels for *Wigglesworthia* genes. (B) Transcript levels for *Glossina* genes. (C) Expression of *pdxB* within tsetse tissues during lactation. Transcript levels were determined by qPCR analysis. The data were analyzed with CFX Manager software, version 3.1 (Bio-Rad). Data represent the means ± SEs for four samples and were normalized to *tubulin*. The asterisk indicates that the expression is significantly higher ($P < 0.05$) than that in newly emerged teneral flies (0 days) or other tissues/samples.

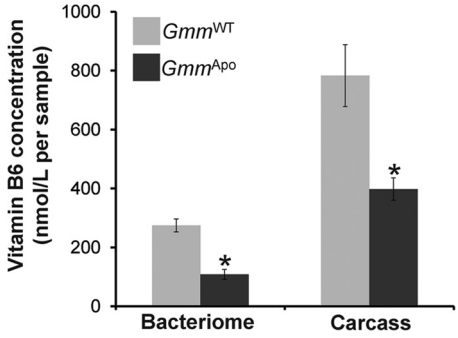

FIG 4 Vitamin B_6 levels in bacteriome and carcass tissues of wild-type and aposymbiotic tsetse. Data represent the means ± SEs of 4 to 5 samples. The asterisks indicate that the expression in WT flies is significantly different from that in Apo flies ($P < 0.05$). *Gmm*, *G. morsitans morsitans*.

abundance within the bacteriome, suggesting that this tissue is likely the prominent site of vitamin B_6 synthesis (Fig. 3C). These results suggest that both the symbiont genes involved in vitamin B_6 metabolism and the host genes involved in salvage functions increase in expression immediately before and during lactation/larvigenesis in mothers.

Vitamin B_6 levels in WT and Apo flies. We examined vitamin B_6 levels in the bacteriome and the corresponding carcasses of WT and Apo females. In Apo females, vitamin B_6 levels were reduced significantly by 40% and 50% in bacteriomes and carcasses, respectively, compared to the same tissues in WT females (Fig. 4). These results confirm that in the absence of *Wigglesworthia*, tsetse contain lower levels of vitamin B_6 in both their whole body and the *Wigglesworthia*-harboring bacteriome.

Proline levels throughout pregnancy in WT and Apo flies. During the lactation cycle in tsetse, transport of amino acids is critical, specifically proline generated by the fat body (17, 19, 42). These amino acids are required for the production of lactation-specific proteins by the milk gland as one of their amino acid constituents and as a source of energy during the process of milk production. Our goal was to determine how this process is altered in Apo mated females. As a baseline, we measured hemolymph proline levels throughout pregnancy in female WT flies. These results show that proline levels increase slightly during the lactation process, decline immediately before birth, and return to prelactation levels within the 3 days following birth. This specific time frame coincides with subsequent embryogenesis of the next offspring (Fig. 5). A similar analysis of proline levels in Apo mated females was performed. These flies normally fail to support the development of larval offspring following oogenesis unless provisioned by dietary supplements (3, 5). We found that proline levels in Apo females increased only minimally during the lactation period relative to WT mated females capable of offspring production (Fig. 5). As expected, the expression of *Wigglesworthia*-specific genes in Apo mated females was reduced by over 98% relative to WT individuals (see Fig. S1 in the supplemental material).

Proline levels following *agat* knockdown with and without vitamin supplementation. RNA interference was utilized to determine if *agat1* expression is critical for maintaining proline levels during tsetse reproduction. Based on qPCR measurements, we were able to demonstrate that treatment with gene-specific short interfering RNA (siRNA) suppressed *agat1* transcript levels by over 80% in pregnant females (see Fig. S2 in the supplemental material). Hemolymph proline levels in WT siAGAT-treated flies were reduced by 30% in comparison to control females that received siGFP (~20 μg proline/μl hemolymph) (Fig. 6A). Hemolymph proline levels in siAGAT-treated females were similar to the levels found in Apo females. Overall, either elimination of the symbionts (Apo females) or suppression of *agat* expression in fertile WT females results in reduced hemolymph proline levels.

Proline levels were next measured from Apo females provisioned with blood diets supplemented with either microbial extracts (yeast or *Wigglesworthia*) or B vitamin cocktails. All three dietary supplementations rescued proline levels in Apo females to that measured in their fertile WT counterparts (Fig. 5B). Supplementation of the blood diet with vitamin B_6 alone also recovered proline levels, but to a lesser extent than did the other treatments. To determine if other B vitamins may also be responsible for the increased proline levels observed in microbial extract-provisioned diets, we supplemented Apo females with all putative *Wigglesworthia*-generated B vitamins (Table 1) except vitamin B_6. We found that this treatment did not increase proline levels significantly (Fig. 6B). Knockdown of *agat* in Apo females followed by yeast or B vitamin supplementation did not lead to a recovery of proline levels compared to control females that received only dietary supplementation (Fig. 6C and D). These results suggest that the ben-

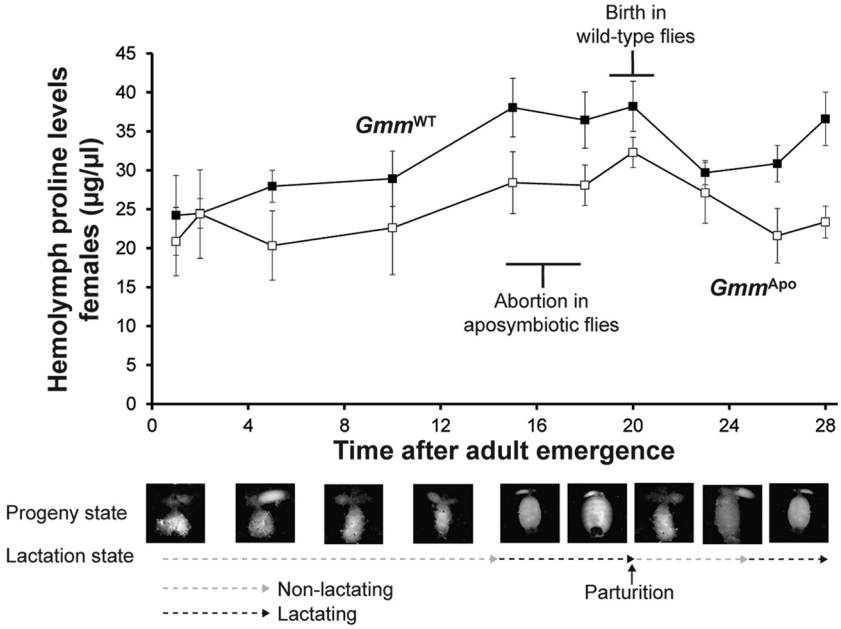

FIG 5 Proline levels in hemolymph throughout lactation from wild-type (WT) and aposymbiotic (Apo) flies. Data represent the means ± SEs for five samples. *Gmm*, *G. morsitans morsitans*.

efits of dietary supplementation in relation to B vitamin synthesis require the presence of AGAT.

Impact of *agat* knockdown on female fecundity with and without vitamin supplementation. After characterizing hemolymph proline levels relative to the dynamics of AGAT and production of vitamins, we next determined the impact that these interactions have upon reproduction. Female fecundity was measured as a function of cumulative larval depositions over the first two gonotrophic cycles. Fecundity of Apo females was reduced by nearly 95% compared to WT flies maintained on a normal blood diet (Fig. 6E). Fecundity in Apo females returned to wild-type levels when these flies were fed a diet supplemented with either yeast or *Wigglesworthia* extracts (Fig. 6E). Supplementation with a cocktail of B vitamins (Apo+VitB flies) resulted in a partial (~50%) recovery of fecundity in Apo flies compared to those that received supplements of either yeast or *Wigglesworthia* cell extracts (Fig. 6E). Dietary provision of vitamin B_6 alone (Apo+VitB6 flies) induced an insignificant recovery in fecundity in Apo females (Fig. 6E). In the case of Apo+VitB−B6 flies, recovery of fecundity was similar to that observed using blood supplemented with only vitamin B_6. Knockdown of *agat* resulted in a substantial reduction in fecundity in WT flies. However, this outcome was not as drastic as the reduction observed in Apo flies (Fig. 6E). Thus, although vitamin B_6 can recover proline levels, a complete vitamin cocktail is required for significant reconstitution of reproductive output.

Proline levels in trypanosome-infected flies. Hemolymph proline levels in male and pregnant female flies that harbored high parasite loads in their guts and mature salivary gland trypanosome infections were measured and compared to uninfected control flies of the same age and physiological status. Hemolymph proline levels in parasitized flies were reduced by almost 50% compared to that found in uninfected individuals of both sexes (Fig. 7).

DISCUSSION

Female tsetse flies must house obligate *Wigglesworthia* bacteria to produce viable offspring. The molecular mechanisms that mediate this aspect of the tsetse-*Wigglesworthia* symbiosis are poorly understood. In this study, we determined that symbiont-provisioned B vitamins regulate critical aspects of host proline homeostasis during the nutritionally demanding periods of tsetse lactation. In the absence of *Wigglesworthia*, Apo females are incapable of maintaining adequate proline levels during early lactation, and this likely contributes to increased larval abortion. However, supplementation of the Apo female diet with yeast extract, *Wigglesworthia* cell extracts, or B vitamins rescues hypoprolinemia and also larval development. We also present evidence that *Wigglesworthia*-produced vitamin B_6 is an essential cofactor for the host enzyme AGAT, which is required to regenerate proline from alanine during proline biosynthesis. Collectively, these results provide the evidence for the role of *Wigglesworthia*-produced metabolites in tsetse physiology. We also show that infections with parasitic trypanosomes, which also utilize proline as the major energy source in tsetse, reduce available hemolymph proline levels. This decline may account for the loss of fecundity observed in parasitized flies (42).

Tsetse is one of a few insects that rely on proline for an energy source, similar to the role of glucose-based molecules in other insect systems (20). In mosquitoes, hemolymph proline is used as a sink for nitrogen during blood digestion (43). Ancestral tsetse's transition to a diet composed exclusively of vertebrate blood (which contains low to moderate levels of sugars but high levels of protein and amino acids) may have coincided with the fly's use of proline as its sole hemolymph nutrient. The regeneration of proline stores is accomplished by the conversion of alanine to proline (3). The first step of this process requires the enzyme AGAT, which employs vitamin B_6 as a cofactor. Our gene expression re-

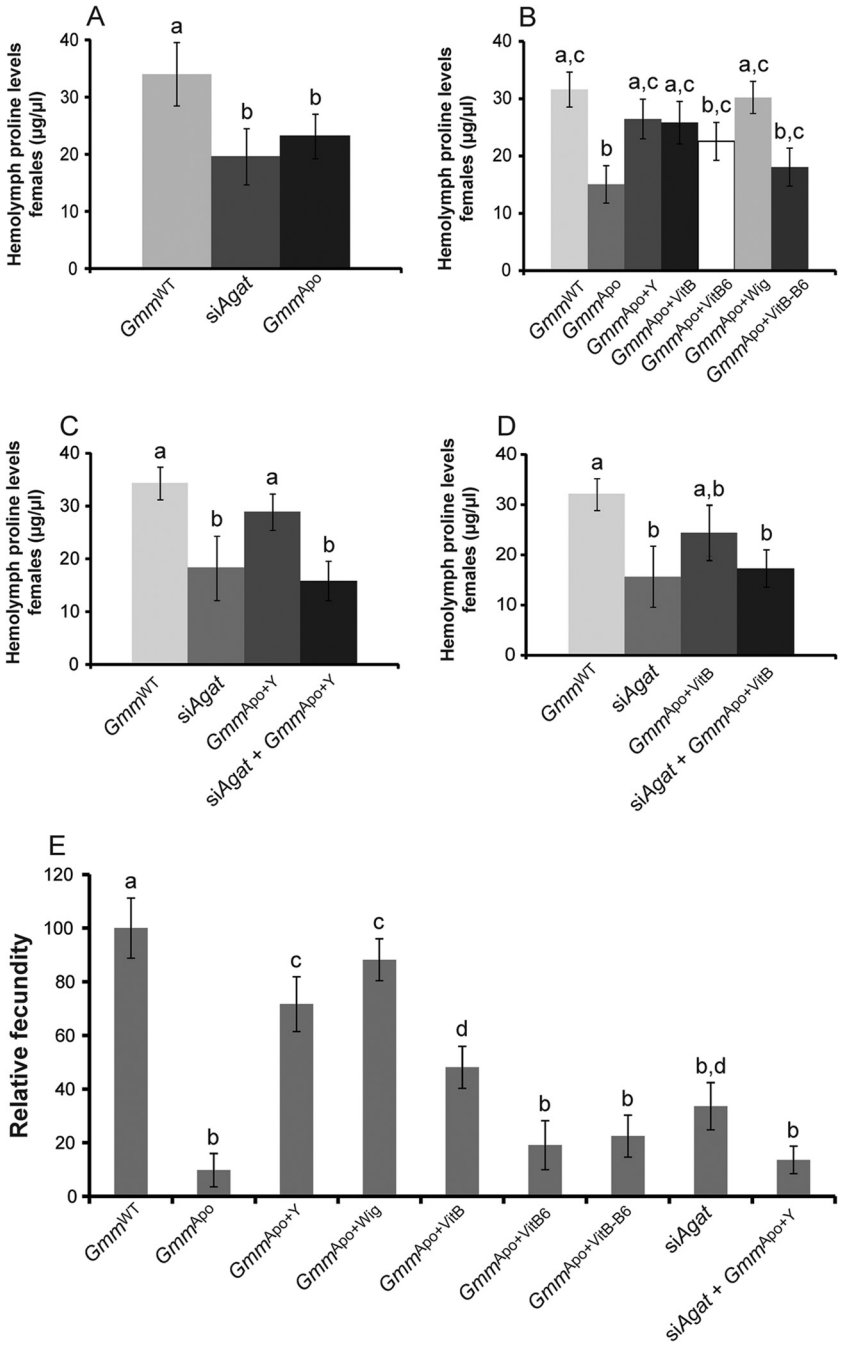

FIG 6 Proline levels in flies after knockdown of *agat* and after supplementation with microbial extracts and B vitamin cocktails. (A) Knockdown of *agat1* through utilization of injection of siRNA. (B) Supplementation of aposymbiotic flies with yeast extract, bacteriome (*Wigglesworthia*) extract, and B vitamins. (C) Knockdown of *agat1* in aposymbiotic flies followed by treatment with yeast extract. (D) Knockdown of *agat1* in aposymbiotic flies followed by treatment with B vitamins. (E) Fecundity following supplementation with microbial extracts and after knockdown of *agat1*. Letters indicate no difference in expression between samples ($P > 0.05$). *Gmm*, *G. morsitans morsitans*.

sults indicate preferential induction for one member of the *agat* gene family, *agat1*, during the period when pregnant females invest heavily in the production of milk to support the intrauterine larval development. Global gene expression analysis of pregnant females indicates that almost 50% of their transcriptional investment corresponds to genes coding for the protein constituents of milk that nourishes intrauterine progeny (12). *agat1* transcripts were among the few non-milk protein-encoding genes that were expressed at higher levels in lactating than in nonlactating flies (12). In addition to *agat1*, expression profiling of vitamin B_6 metabolism-related genes carried by *Wigglesworthia* shows coordinately induced expression during pregnancy when nutritional demands for larval growth are high. Measurement of proline levels during the different stages of pregnancy shows a concurrent in-

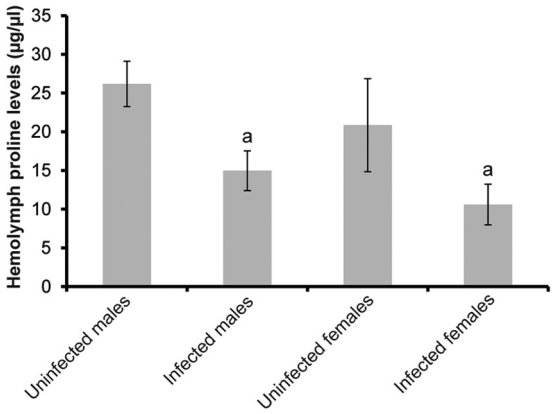

FIG 7 Proline levels in flies infected with trypanosomes. Data represent the means ± SEs for nine samples. "a" indicates that the proline levels in uninfected and infected flies are significantly different ($P < 0.05$).

crease during periods of lactation. When host vitamin-related genes, as well as *agat1* expression, were measured from *Wigglesworthia*-lacking Apo adults, the lactation-associated regulation profile was not observed. In addition, a reduction in proline levels in Apo adults that were unable to support larval development was observed in comparison to fertile WT females. The results from experiments in which we suppressed *agat* levels in fertile WT females mimic the reduction in hemolymph proline levels observed in Apo females. Collectively, these results indicate that AGAT functions are critical for the maintenance of proline homeostasis, especially during periods of pregnancy/lactation in tsetse.

Dietary supplementation experiments in Apo adults showed that microbial extracts, as well as a complete mixture of B vitamins, could restore proline levels to those found in fertile WT females. Dietary supplementation of Apo flies with a vitamin mixture lacking vitamin B_6 did not rescue proline levels, further demonstrating the role of vitamin B_6 in host fecundity. In addition, hemolymph proline levels of Apo adult females could not be rescued by dietary supplementation when *agat1* expression was reduced by siRNA treatments. Thus, symbiont-provisioned vitamins facilitate AGAT enzymatic function to allow proline regeneration/homeostasis. More so, vitamin B_6 in particular is a particularly important component of the symbiotic contributions.

We also evaluated the effect of vitamin provisioning on host fecundity by measuring cumulative female larval deposition over the two gonotrophic cycles under different treatments. Dietary supplementation of Apo females with microbial extracts shows significant rescue effects of about 80% of WT flies but not WT flies treated with siAGAT. Similar dietary provisioning with complete B vitamin cocktails, which required vitamin B_6, rescued fecundity. Supplementation of vitamin B_6 alone did not recover fecundity. Likely, the role that symbionts play in terms of tsetse fecundity involves provisioning of multiple factors, including a B vitamin cocktail, with the facilitation of vitamin B_6 to promote proline homeostasis playing a major role.

Outside tsetse, the role of the B vitamin complex has been studied in multiple organisms, but many of these studies have been limited to the examination of fecundity and other basic physiological characteristics such as adult emergence (5, 37, 44). For obligate blood-feeding insects such as bedbugs and lice, symbionts are presumed to provide B vitamins in addition to those found within the blood (4, 5, 37). Examination of the role for B vitamins is limited to removal of the symbiont through antibiotic treatment and subsequent recovery of impaired fecundity and development by dietary supplementation with excess B vitamins (3–5, 37). Recently, Nikoh et al. (44) have demonstrated that vitamin B_7 (biotin) produced by their mutualistic *Wolbachia* is absent following antibiotic treatment, leading to impaired adult emergence. This impaired emergence can be recovered by a cocktail of B vitamins that requires biotin (44). Here, we show a link between a metabolic process (proline synthesis) and a specific B vitamin (vitamin B_6) that is at least partially responsible for the reduced fecundity. Interestingly, vitamin supplementation failed to rescue other impaired physiological processes, such as immune system development, that are present when tsetse lack *Wigglesworthia* (35, 36). Thus, other factors, such as immune priming by *Wigglesworthia*, critical to tsetse health and development are likely due to factors or mechanisms associated with *Wigglesworthia* other than B vitamin synthesis.

The tsetse flies utilized in this study also harbor *Sodalis*, and this bacterium's genome carries the genes required for vitamin B_6 production (45). *Sodalis* is not present in all tsetse populations and, when present, represents less than 0.5% of the cumulative gut bacterial population (*Wigglesworthia* accounts for over 99% of the bacterial species) (46). Thus, although both endosymbionts are present, *Wigglesworthia* is likely responsible for producing most, if not all, vitamin B_6 required to maintain physiological processes.

Parasite infections in various insects result in loss of fecundity rather than reduced longevity (47, 48). This outcome is logical since parasite transmission to the next mammalian host depends more upon host vector insect survival and feeding on multiple hosts than upon producing an optimal number of offspring (47–49). Tsetse females infected with a highly immunogenic trypanosome strain exhibited decreased fecundity, although these flies lived equally as long as did uninfected tsetse or tsetse infected with a nonimmunogenic parasite strain (47). The decline in fecundity observed in tsetse females infected with the immunogenic parasite strain can be attributed to the physiological cost of host immune system activation (47). Trypanosomes also use proline as the major carbon source during their development in tsetse (47–49). Here, we demonstrate that parasitized tsetse also present significant reductions in hemolymph proline levels. Thus, parasite infections may be costly to tsetse reproduction either because they compete for available host proline or B vitamins or because tsetse flies utilize more proline in processes such as the induced immune response associated with immunogenic parasite infections.

We have developed a putative model that defines the association between *Wigglesworthia*, tsetse, and proline homeostasis relative to the fly's reproductive cycle (Fig. 8). Before lactation, B vitamins, specifically vitamin B_6, are present at levels necessary for tsetse to maintain proline homeostasis. Upon pregnancy-induced lactation, proline biosynthesis increases and the amino acid is taken up by the milk gland as an energy source for nutrient production. In the case of aposymbiotic tsetse that lack *Wigglesworthia*, blood vitamin levels, particularly those of vitamin B_6, are insufficient to fuel AGAT functionality. Loss of AGAT function disrupts proline homeostasis such that larval growth cannot be fully supported. Hence, Apo females without proper dietary supplementation abort developing larvae and exhibit reduced fecundity. However, they do appear capable of completing oogenesis and embryogenesis, which are much less metabolically demand-

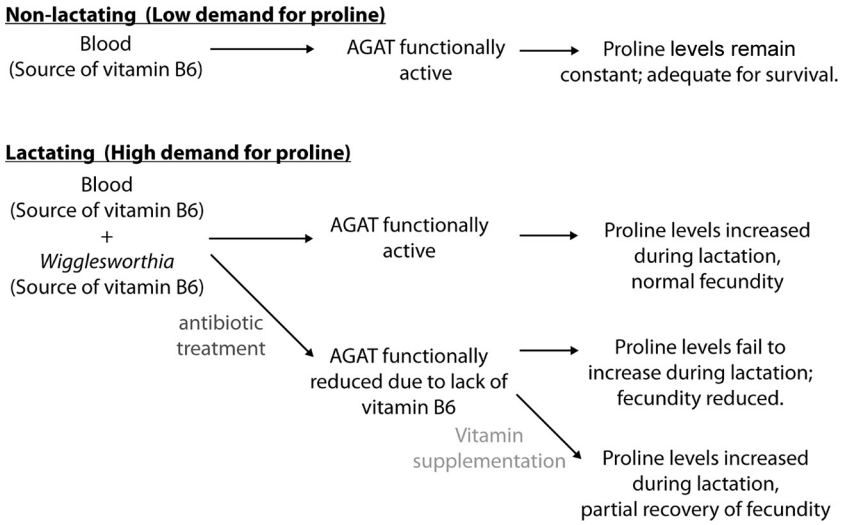

FIG 8 Summary of the role of vitamin B_6 in relation to proline homeostasis.

ing than lactation/larvigenesis. Furthermore, reduced fecundity noted in parasitized flies appears to result from host-parasite competition for essential nutrients in addition to the cost of host immune response inductions.

Conclusions. This study directly links a vitamin produced by an obligate symbiont (*Wigglesworthia*) to the function of a specific insect host (tsetse) metabolic pathway. We show that *Wigglesworthia*-produced vitamin B_6 is critical for the prevention of hypoprolinemia, specifically during lactation when proline is required both as a blood-borne nutrient resource and as a key amino acid for milk production. Interestingly, vitamin B_6 alone does not recover fecundity completely, suggesting that the multiple B vitamins produced by *Wigglesworthia*, including folate and thiamine, likely also have distinct roles in relation to tsetse physiology and reproduction. In addition, since vitamin B_6 is a required factor for most transamination reactions, it may also be involved in other processes beyond acting as an AGAT cofactor that are critical to tsetse biology.

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