

Sphingomyelinase Activity in Mother's Milk Is Essential for Juvenile Development: A Case from Lactating Tsetse Flies¹

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ABSTRACT

Sphingosine is a structural component of sphingolipids. The metabolism of phosphoethanolamine ceramide (sphingomyelin) by sphingomyelinase (SMase), followed by the breakdown of ceramide by ceramidase (CDase) yields sphingosine. Female tsetse fly is viviparous and generates a single progeny within her uterus during each gonotrophic cycle. The mother provides her offspring with nutrients required for development solely via intrauterine lactation. Quantitative PCR showed that acid *smase1* (*asmase1*) increases in mother's milk gland during lactation. aSMase1 was detected in the milk gland and larval gut, indicating this protein is generated during lactation and consumed by the larva. The higher levels of SMase activity in larval gut contents indicate that this enzyme is activated by the low gut pH. In addition, *cdase* is expressed at high levels in the larval gut. Breakdown of the resulting ceramide is likely accomplished by the larval gut-secreted CDase, which allows absorption of sphingosine. We used the tsetse system to understand the critical role(s) of SMase and CDase during pregnancy and lactation and their downstream effects on adult progeny fitness. Reduction of *asmase1* by short interfering RNA negatively impacted pregnancy and progeny performance, resulting in a 4–5-day extension in pregnancy, 10%–15% reduction in pupal mass, lower pupal hatch rates, impaired heat tolerance, reduced symbiont levels, and reduced fecundity of adult progeny. This study suggests that the SMase activity associated with tsetse lactation and larval digestion is similar in function to that of mammalian lactation and represents a critical process for juvenile development, with important effects on the health of progeny during their adulthood.

ceramide, *Glossina*, lactation, milk, sphingomyelin

INTRODUCTION

Sphingolipids are critical components of cell membranes of eukaryotes and some bacteria [1–5]. These lipids not only are necessary for cell membrane integrity, but are critical for cell signaling [1–5]. Sphingomyelinases (SMases) catalyze the hydrolysis of phosphocholine (PC)-ceramide (mammalian sphingomyelin) to phosphocholine and ceramide in mammals [1–3]. Following the breakdown of sphingomyelin, ceramide

can be metabolized to sphingosine and free fatty acid by ceramidase (CDase) [6, 7]. The presence of SMase and CDase during digestion is critical for the provision of sphingomyelin-based lipids from food sources for cell membrane development and sphingosine-based cell signaling processes [3, 8, 9].

In humans, defects in SMase encoding genes causes Niemann-Pick disease, a neurodegenerative disorder resulting in death before 3 years of age in severe cases [2, 10, 11]. Insects differ from mammals in that SMase hydrolyzes phosphoethanolamine (PE)-ceramide (insect sphingomyelin) to yield PE and ceramide. Although the substrate differs between the mammalian and insect SMases in the chemical moiety attached to the ceramide, the basic structure and function of the SMase enzymes and their metabolic products are similar [3]. Thus, insects could serve as model systems for studying metabolic diseases related to sphingomyelinase deficiency [2, 3].

SMases are classified according to the pH at which they are activated; acid SMases (aSMases) function at low pH, neutral SMases (nSMases) near pH 7, and alkaline SMase near pH 9 [2, 3]. Few studies have addressed the role of SMases in relation to insect physiology [3]. In *Drosophila*, *asmases* and *nsmase* are widely expressed during embryonic development [1]. Microarray studies revealed that an *asmase* (CG15533) and neutral *cdase* are downregulated in *Drosophila* after sugar feeding and may play a role in suppressing fatty acid synthesis [12].

In tsetse (*Glossina morsitans morsitans*), lipid metabolism plays a major role in the reproductive cycle [13–16]. Tsetse reproduction is viviparous. The female tsetse carries a single intrauterine larva and the only source of essential nutrients for immature development is female milk products generated from the accessory gland. This is drastically different than most insects, which are oviparous, and deposit many eggs during each gonotrophic cycle. About 50% of the nutrients in the tsetse milk are lipids [13–16]. The evolution of viviparity has driven dramatic adaptations in the tsetse's reproductive physiology [17]. These modifications include reduced ovary capacity and production (a single oocyte maturing during each gonotrophic cycle), increased uterine capacity (capable of accommodating a developed third instar larva equal in mass to the mother), and expansion of the female accessory gland into a lactating organ (milk gland) to provision nourishment to the intrauterine progeny via its secretions [17–20]. The first progeny is deposited 20–22 days post-adult eclosion, with each subsequent larva being deposited every 10–11 days thereafter [17]. This slow reproductive rate limits tsetse mothers to 8–12 births of single progeny in their lifetime [17]. Thus, interference with tsetse pregnancy could drastically reduce tsetse populations.

The only source of nutrients for adult tsetse is vertebrate blood. Blood is extremely rich in some nutrients such as

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proteins and lipids, but nearly devoid of essential vitamins and other micronutrients. These lacking nutrients are provided to tsetse flies by their obligate symbiont, *Wigglesworthia glossinidia* [21]. Specifically, this bacterium is thought to produce B vitamins that are critical for many biochemical processes [21]. It is important to note that presence of this symbiont is critical to tsetse reproduction because elimination of the bacteria via antibiotic treatment leads to a significant reduction in fecundity [22].

Lactation in tsetse and mammals has evolved independently, but essential requirements inherent to the lactation process appear to be conserved between the two systems. These include the development of specialized secretory cells in the lactating tissue, high lipid content and composition of the milk, functional conservation of milk proteins (i.e., lactoferrin and lipocalins in mammals and transferrin and milk gland protein in tsetse, respectively), and transfer of beneficial symbiotic bacteria from mother to progeny via milk secretions [19, 23–31]. Thus, tsetse could represent a novel model system for the study of the mechanisms underlying lactation.

Previous work showed that mammalian milk secretions contain an activated SMase function, which is stimulated during digestion in newborns [7, 32, 33]. Here, we first analyze SMase function(s) during tsetse fly pregnancy and lactation to determine if this enzyme is fulfilling an orthologous function. We report on the spatial and temporal expression of *smases* and *cdases* throughout tsetse pregnancy. We next use an RNA interference-based gene silencing approach to evaluate the functional roles of these enzymes in pregnancy and the downstream impact of their modification on the health of the progeny. Our findings suggest that the presence of SMase in tsetse milk is critical for the provision of essential sphingosine products to intrauterine larva during development. Modification in SMase activity within tsetse milk negatively impacts female fecundity and the health of progeny. We discuss the role of tsetse as an important invertebrate model with which to study the role of sphingolipids and SMases during lactation.

MATERIALS AND METHODS

Flies

Glossina morsitans morsitans, utilized at Yale University and the Slovak Academy of Sciences, originated from a population collected in Zimbabwe and had been in culture for at least 20 years. Flies were maintained at 25°C and 50%–60% RH using an artificial membrane feeding system [34]. Females were mated 3–5 days after emergence. Flies were collected according to established developmental markers based on oocyte, embryo, and larva presence [29, 34].

Phylogenetic Analysis of Tsetse *smase* and *cdase*

BLASTX analysis of the tsetse genome using *Drosophila* sequences was utilized to identify the tsetse genomic scaffold containing the *smase* and *cdase* sequences. Full length sequences were obtained by mapping Illumina high-throughput reads against identified genomic scaffolds using the CLC Genomics software package (CLC Bio). Predicted protein sequences were aligned using ClustalX [35] and formatted with BioEdit [36]. Pairwise phylogenetic tree construction and bootstrap analysis (10 000 replicates) were performed using the MEGA5 sequence analysis suite [37].

RNA and Protein Extraction

RNA and protein were extracted from whole flies and tissues using Trizol reagent according to the manufacturer's protocol (Invitrogen). Complementary DNA was synthesized from 1 µg of total RNA from two combined flies using Superscript III reverse transcriptase kit (Invitrogen) based on the manufacturer's protocol, with the exception that cDNA synthesis was extended to 60 min. RNA and cDNA were stored at –70°C until use. Protein was stored in protein pellet solubilization buffer (8 M urea, 3 M thiourea, 1% dithiothreitol, and 4% CHAPS) at –20°C.

Analysis of *smase* and *cdase* Expression

Levels of *asmase1–4* (JQ308535, JQ308537–JQ308539), *nsmase* (JQ308535), *cdase* (JQ308540), brain washing (*bwa*, a protein with potential CDase-like activity, JQ308541), milk gland protein 1 (*mglp1*, a lipocalin that is expressed highly during tsetse pregnancy [13], DQ294227.1) and lipophorin receptor (*lpr*, a gene utilized as a control because it varies little throughout tsetse pregnancy, DQ294226.1) were determined by quantitative PCR (qPCR) utilizing the iCycler iQ real-time PCR detection system (Bio-Rad) using gene-specific primers (Supplemental Table S1; all Supplemental Data are available online at www.biolreprod.org). The data were obtained in triplicate samples and were normalized to tsetse tubulin (*tub*, DQ377071.1) expression levels and analyzed with CFX Manager software version 3.1 (Bio-Rad).

Knockdown of SMase

Short interfering RNA (siRNA) consisting of two Duplex sequences (CAGCAAUAAUUUCAACUUGGUGAUACUC and AAAUAAUUCAAAGCUUUGUUCACCAA) was obtained commercially (IDT). Control siRNA were designed against green fluorescent protein (GFP; GAUGCCAUUCUUUGUUUGUCUCCAU and CUUGACUUCAGCAGUGUCAUUGUAGUU). Previous studies on insects, and specifically tsetse, have demonstrated gene knockdown by injection of siRNA [16]. The siRNA concentration was determined by spectrophotometer and adjusted to 700–750 ng/µl. Each fly was injected with 1.5 µl siRNA 8–10 days after adult emergence. Previous studies show that injection of siRNA into the mother has no discernable effect on larval transcript levels [13]. Expression levels were determined by qPCR (as described) 5 days after siRNA injection and normalized to *tubulin*. Mass of the pupae was determined utilizing an electrobalance (Cahn 25) and wing length was measured by the Comstock-Needham method [38]. For pupal hatch assessment, pupae were held in a Petri dish for 60 days and the number of adults that emerged was measured. Fecundity of progeny from mothers with reduced aSMase1 was compared to that from mothers injected with siGFP and mothers injected with siMilk gland protein (siMGP) for comparative analysis to previous studies [29]. Pupae where no adults emerged were dissected to determine if development had ceased and mortality occurred.

Heat Tolerance

Within 24 hours of emergence teneral female offspring of aSMase1 knockdown flies were subjected to 40, 42.5, and 45°C in groups of 10 for 2 h. Subsequently, the flies were returned to normal colony rearing conditions at 25°C. One day postexposure to heat challenge, flies were analyzed and survival was indicated when an individual responded to mechanical disturbance. Each experiment was replicated three times.

Symbiont Levels Within Teneral Flies and Expression of *Wigglesworthia BioF*

Symbiont genome numbers were quantified by qPCR using the *groEL* (AF321516.1) primer set specific for obligate *W. glossinidia*, with data normalized to host *tubulin* [39]. BioF is an enzyme involved in biotin synthesis [21], but is structurally similar to serine palmitoyl transferase (SPT), which is an enzyme involved in the sphingolipid biosynthetic pathway [4]. Transcript levels for BioF were determined in the bacteriome (the organ that harbors the symbiont in the midgut), in the adult carcass (which has milk gland tissue), and in larval gut contents (where *Wigglesworthia* transferred in the milk is exposed to sphingosine products). Levels of *bioF* were normalized according to *groEL*, which was shown to be consistently expressed in the bacteriome, mother, and larvae [39]. Total RNA was extracted from five teneral female flies within 48 h of adult emergence and cDNA for qPCR analysis was synthesized as described previously. qPCR primers utilized in this section are provided in Supplemental Table S1.

pH Determination of Milk Gland, Uterine Fluid, and Larval Gut Contents

Milk gland, uterine fluid, and larval gut contents were collected from females pregnant with late second/early third instar larvae. Females were immobilized on ice and the reproductive tract was exposed by dissection. Uterine fluid was collected using a 20-µl pulled glass capillary needle from within the uterus using negative pressure according to Benoit et al. [13]. Milk glands were removed at the attachment site to the uterus, separated from the fat body, and stored in groups of 10 at –20°C until treatment. Individual larvae were obtained from the uterus by removing the upper portion of the reproductive tract and extracting the progeny with soft-tip forceps. Larval guts

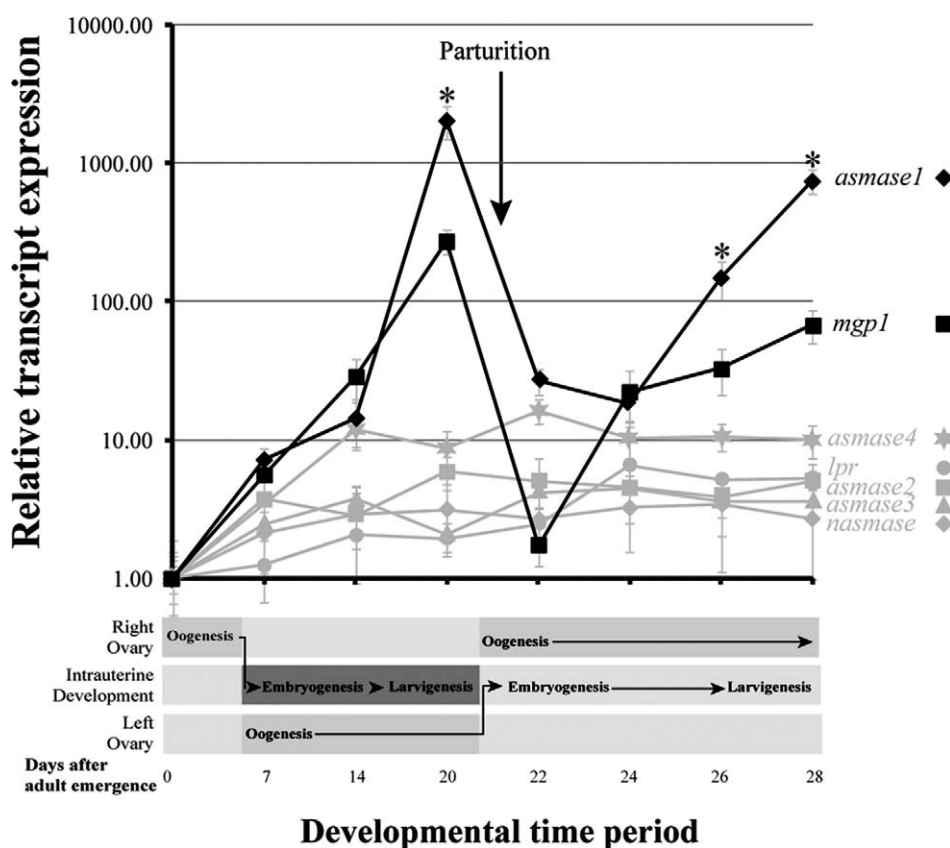


FIG. 1. Time course of *asmase1–4* and *nsmase* expression during the first two tsetse gonotrophic cycles. Milk gland proteins 1 (*mgp1*) and lipophorin receptor (*lpr*) transcript levels are shown for comparative purposes. Transcript levels were determined by qPCR utilizing the iCycler iQ real-time PCR detection system (Bio-Rad). The data were analyzed with software version 3.1 (Bio-Rad). Data represent the mean \pm SEM of three replicates and were normalized to tubulin. Significant differences (* $P < 0.01$) were determined by ANOVA followed by Tukey test in comparison to samples that do not increase in relation to pregnancy.

were removed by dissection. Milk gland tissue and larval guts were macerated without buffer and centrifuged at 2000 rpm. The supernatant was removed and pH was tested with a microprobe (Sigma-Aldrich). Each tissue type was replicated five times and represents combined tissue from five flies.

SMase Activity

SMase activity was determined by a standard fluorescent assay (SMase Enzymatic Assay; Caymen Chemical). Milk gland, uterine fluid, and larval gut contents were collected as described. Samples were analyzed without buffer (to maintain the physiological pH within the organs) or buffered to pH 6.0 with citrate buffer or 7.8 with PBS according to manufacturer's protocol. Samples were stored at -20°C until use. Each sample was measured using a fluorescent plate reader (Biotek) with an excitation wavelength of 535 nm and absorbance at 590 nm. Activity was determined by comparison to a phosphocholine standard. Each tissue type was replicated three times and each replicate represents combined tissue from five flies. Protein concentrations were standardized according to total protein using a Bradford assay.

Western Blot Analysis

Proteins from each tissue represent combined samples from at least 10 flies. Rabbit antisera against tubulin (GmmTub) were previously described [29] and rabbit polyclonal human-SMase antisera were commercially obtained (Abcam). Blots were blocked overnight in PBS, 3% bovine serum albumin, and 0.5% Tween 20 (blocking buffer). The aSMase antibodies were used at 1:1000 concentration. The equivalent of 1/400 of a fly was loaded into each well. Blots were visualized with Supersignal West Pico Substrate (Pierce) on an Image Station 2000R (Kodak).

Immunohistochemistry of SMase

Immunohistochemical analysis was performed with aSMase antisera in blocking buffer (1:200). Goat anti-rabbit IgG conjugated with DyLight 488

(Thermo Scientific) was used as the secondary antibody at 1:500 concentration. Tissues from pregnant flies were dissected, fixed, and stained as described [29, 40].

RESULTS

Tsetse SMase and CDase Genes Are Orthologous to Those Found in *Drosophila*

Sequences for five *smases* (*asmase1*, *asmase2*, *asmase3*, *asmase4*, and *nsmase*) and two *cdases* (*cdase* and *bwa*) were identified from tsetse fly genome data via BLAST using *Drosophila* SMase protein sequences (Supplemental Fig. S1). Structural analysis of all five predicted tsetse SMases highlighted the presence of a conserved SMase metallophosphatase domain in each of the sequences. Phylogenetic analysis of the predicted tsetse SMases (four aSMases and one nSMase) with *Drosophila* SMases revealed that each tsetse sequence is orthologous to individual *Drosophila* sequences (Supplemental Fig. S1). The percentage identity and similarity between the tsetse and *Drosophila* SMases range from 50% to 73% and 69% to 84% respectively. A search for CDase genes using a similar approach as described above yielded two genes. Although there are no defined domain structures associated with CDases, the tsetse genes show high homology to characterized CDases in *Drosophila*. Each of the two tsetse CDases has representative orthologs in *Drosophila* (CDase and Bwa; Supplemental Fig. S2). These data suggest that the SMase and CDase enzyme families are conserved between *Drosophila* and tsetse.

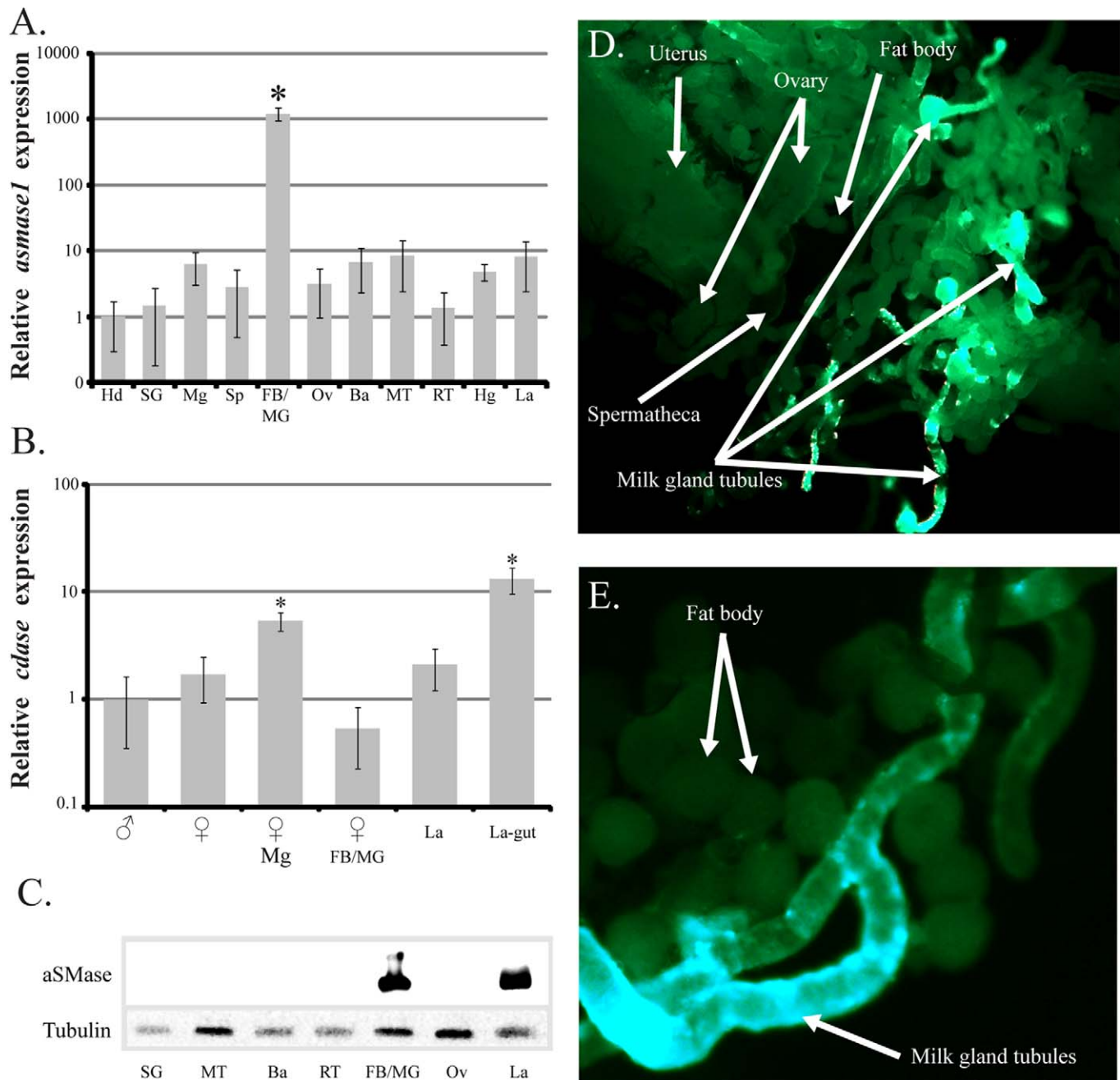


FIG. 2. Spatial analysis of transcript expression and immunolocalization of SMase. **A** and **B**) Acid SMase 1 (*asase1*; **A**) and CDase (*cdase*; **B**). Transcript levels were determined by qPCR using the iCycler iQ real-time PCR detection system (Bio-Rad). The data were analyzed with software version 3.1 (Bio-Rad). Data represent the mean \pm SEM for three samples and was normalized to *tubulin*. *Significant difference determined by ANOVA followed by Tukey test ($P < 0.01$). **C**) Western blot analysis of SMase tissue distribution. Tsetse female midgut contents were not tested because of cross-reaction of the human SMase antibody and SMase present within the cow blood utilized to feed the flies. Hd, hindgut; SG, salivary gland; Mg, midgut; Sp, spermatheca; FB/MG, fat body/milk gland; Ov, ovaries; Ba, bacteriome; MT, malpighian tubules; RT, reproductive tract; Hg, hindgut; La, larvae. **D**) Immunolocalization of SMase in reproductive tract during pregnancy. **E**) Immunolocalization of SMase in fat body and milk gland. Images are representative of three separate tissue stainings.

Tsetse *aSMase1* Is Regulated and Expressed in a Pregnancy- and Milk Gland-Specific Manner

The expression profile of the tsetse *smase* and *cdase* genes was followed in fertile females over the course of the first two gonotrophic cycles by qPCR analysis. Within the first 24–48 hours of adult emergence, transcripts for *asase1–4* and *nsmase1* increased to a baseline level. Levels of *asase2–4* and *nsmase* remained constitutive for the remainder of the time course (Fig. 1). In contrast, levels of *asase1* increased by over

1000-fold during pregnancy/lactation (13–20 days), but declined rapidly to baseline levels after birth (parturition at 20 days). This expression pattern for *asase1* was repeated again during the second gonotrophic cycle (Fig. 1). The *asase1* expression profile matched that of the major milk protein (*gmmmgp1*), which is a lactation-associated lipocalin. The expression of *gmmmgp1* was found to increase over 100-fold during pregnancy/lactation, but was down dramatically within 48 h after parturition (Fig. 1). A recently completed

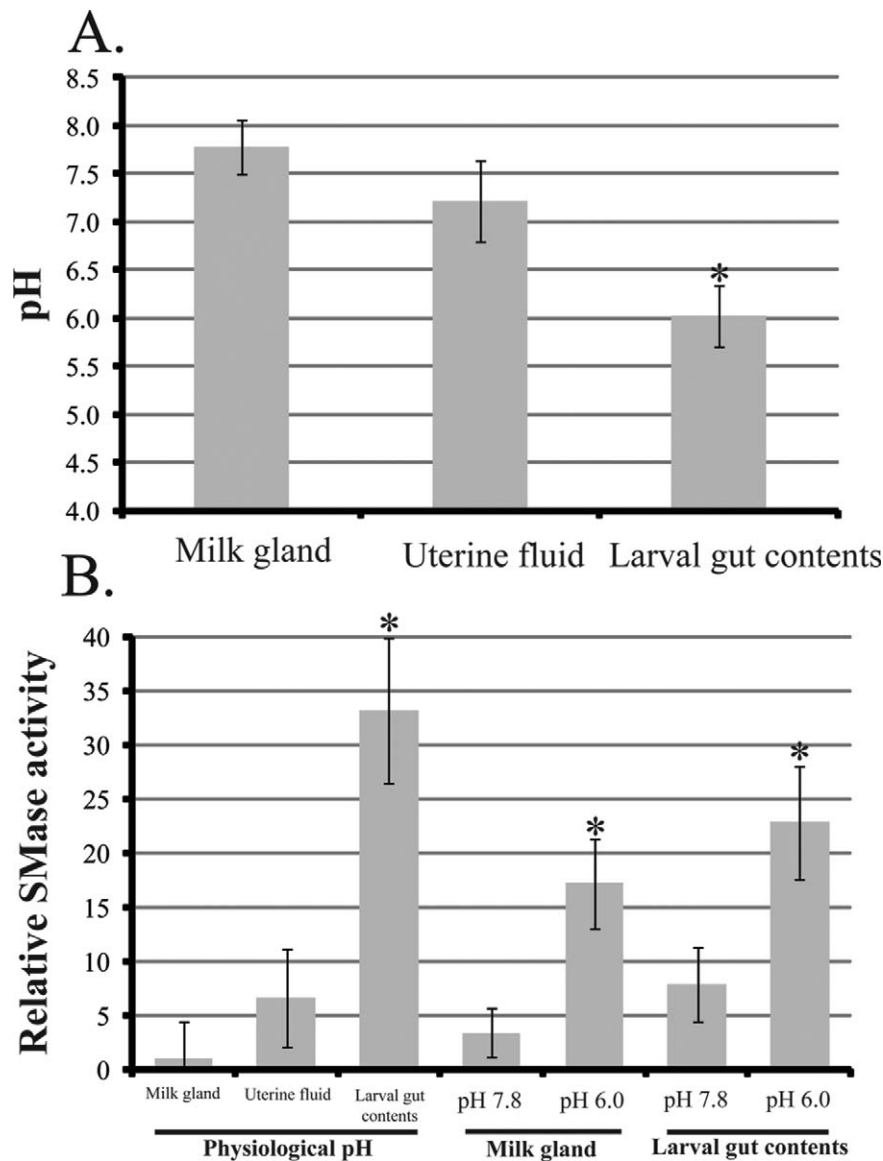


FIG. 3. The pH and SMase activity in milk gland tissue, uterine fluid, and larval gut content of pregnant tsetse flies and their larval offspring. **A** and **B** pH, mean \pm SEM of five measurements (**A**), and activity of SMase, mean \pm SEM of three measurements (**B**). The mean physiological pH for milk gland is 7.8, uterine fluid is 7.2, and larval gut contents is 6.0. Milk gland and larval gut content were buffered to pH 7.4 with PBS and to pH 6.0 with sodium citrate buffer. *Significance following ANOVA with Tukey test at $P < 0.01$.

high-throughput sequencing project, comparing the transcriptome of pregnant versus postpregnancy samples, confirmed that *asmase1* is the only SMase gene that increased in expression in pregnant flies compared to those that had undergone parturition (Supplemental Fig. S3). Transcripts for *cdase* or brain washing (*bwa*), a protein with potential CDase activity [40], increased within 24–48 h of adult emergence, but did not vary throughout pregnancy. This is similar to what is observed with the lipophorin receptor (*lpr*), which was previously shown to vary little throughout pregnancy [13] (Supplemental Fig. S4).

Spatial analysis of *asmase1* expression revealed that this gene is preferentially expressed in the milk gland/fat body of pregnant mothers, although some transcripts could be detected in the bacteriome and malpighian tubules as well. In contrast, *cdase* is expressed in the guts of the developing intrauterine larvae and mother (Fig. 2, A and B). Western analysis of SMase proteins indicated the presence of high levels of these

proteins primarily in the milk gland/fat body and larvae (Fig. 2C). Whole mount immunohistochemical staining further confirmed that SMase proteins are localized in the milk gland tubules of lactating mothers (Fig. 2, D and E). This localization profile is consistent with proteins generated in the mother and transferred in the milk to the nursing larva [29, 30].

SMase Enzymatic Activity in Milk Secretions Is Regulated by Environmental pH

The pH of the milk gland, uterine fluid, and larval gut contents was determined to be 7.8, 7.2, and 6.0, respectively (Fig. 3A). SMase activity in the larval gut was over 30 times higher than that measured from the milk gland and uterine fluid (Fig. 3B). However, when the pH of the milk gland contents was decreased to that of the larval gut contents (pH 6.0), SMase enzymatic activity increased by 5–6-fold (Fig. 3B). In addition, when the larval gut contents were buffered to milk gland pH (pH 7.8), SMase activity in the larval contents was

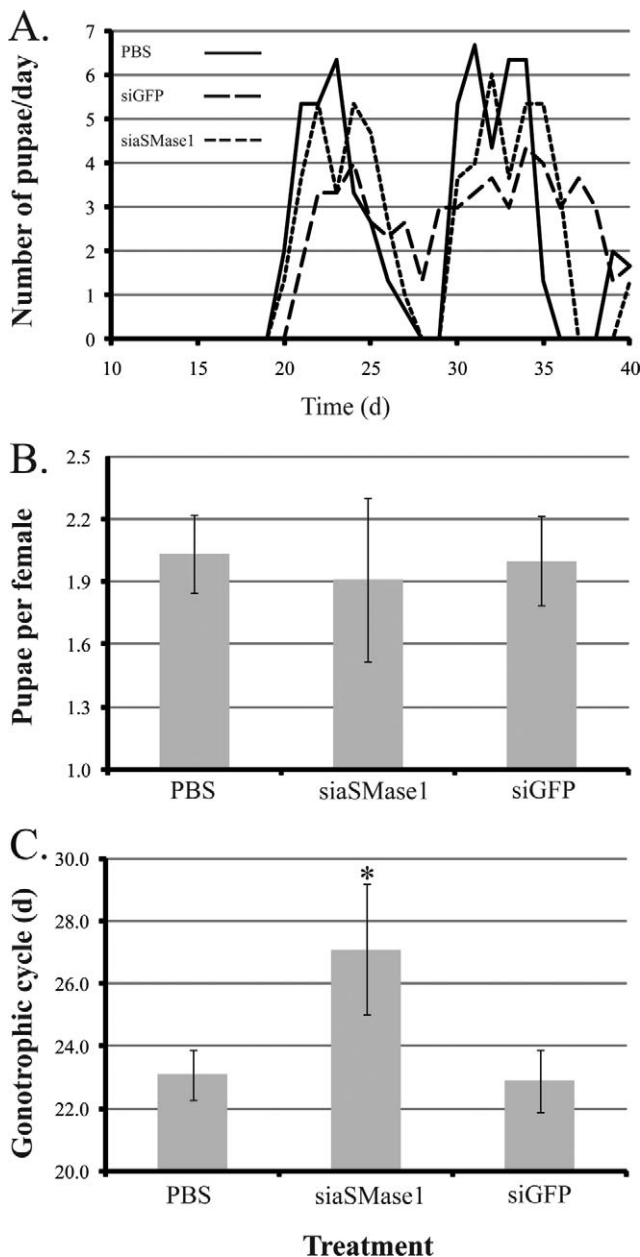


FIG. 4. Phenotype of pregnant female tsetse following injection of aSMase1-specific siRNA. **A**) Daily deposition rates for a representative group of 30 females over 40 days. **B**) Number of pupae per female over 40 days, mean \pm SEM of three groups of 30 flies. **C**) Duration of pregnancy, mean \pm SEM of three groups of 15 flies. *Significant difference from siGFP-injected control following ANOVA with Tukey test at $P < 0.01$.

reduced by 3–4-fold (Fig. 3B). These results indicate that milk SMase is activated by the low pH environment of the larval gut contents. This system likely prevents the premature digestion of sphingomyelins during synthesis/storage in the milk gland and facilitates digestion and uptake of these nutrients within the digestive tract of developing larva.

RNAi Knockdown of aSMase1 Extends Larval Development and Negatively Affects Offspring Health

To determine the role of aSMase1 during pregnancy and larval development, pregnant females were treated with siRNA targeting aSMase1 and subsequently observed for phenotypes associated with pregnancy and offspring performance. Quan-

titative PCR of *asmase1* after siaSMase1 treatment showed an approximately 60% decrease relative to transcript levels in control siGFP-treated flies. Accordingly, SMase activity in the larval midgut was reduced by 50%–60% in progeny from siaSMase1-treated mothers (Supplemental Fig. S5). The total number of pupae produced from control and *asmase1* knockdown flies did not differ over the two gonotrophic cycles analyzed (Fig. 4, A and B). However, the duration of pregnancy was extended by 4–5 days in the *asmase1* knockdown group (Fig. 4C). These results suggest that aSMase1 activity is an important component of the milk for larval development as its reduction causes a significant delay in larvigenesis.

We evaluated the impact of *asmase1* knockdown in the mother on her emerging progeny. The mass of the pupae from siaSMase1-treated mothers measured 5 days after deposition was lower by 1–2 mg (5%–8% reduction compared to the controls; Fig. 5A) and hatch rate of the pupae from *asmase1* knockdown flies was suppressed by 15%–20% (Fig. 5B). The siaSMase1 knockdown in the mothers caused no significant differences in the wing length of emerging adult progeny compared to the controls (0.77 ± 0.04 after siaSMase1 treatment vs. 0.75 ± 0.05 after siGFP treatment). However, heat tolerance of emerging adult progeny from aSMase1 knockdown mothers was negatively impacted at 45°C , although not at 40 or 42.5°C (Fig. 5C). The impaired heat tolerance of adult progeny from siaSMase1 mothers returned to normal levels when analyzed after a blood meal (Fig. 5C). We also evaluated the long-term effects of acquiring reduced SMase1 levels during juvenile development by measuring the fecundity of the emerging adult progeny. Progeny from siaSMase1 mothers produced fewer pupae compared to siGFP controls, although this reduction was not as drastic as suppression of MGP in pregnant mothers (Fig. 5D). Additionally, pregnancy duration was extended in flies from siaSMase1-deficient mothers by 2–3 days (21.9 ± 0.7 days after siGFP treatment vs. 23.9 ± 0.8 days after siaSMase1 treatment). These results indicate that reduction of aSMase1 in milk has significant negative effects upon pregnancy duration along with reduced progeny emergence, progeny thermal stress tolerance, and progeny reproduction output.

SMase Levels Impact Density of the Obligate Symbiont, *Wigglesworthia*, in Emerging Progeny

We assessed if aSMase1 reduction in mother's milk impacted the levels of the obligate *Wigglesworthia* transmitted to the intrauterine larva. This symbiont is required for females to maintain fecundity and is transferred to the developing larva in the milk [19, 22]. Suppression of *asmase1* in the mother resulted in reduced density levels of *Wigglesworthia* (40%–50% reduction compared to controls) in emerging progeny (Fig. 6A). Given the negative effect maternal SMase knockdown has on *Wigglesworthia* transmission to offspring, we measured the expression of a *Wigglesworthia* gene, *biof*. We reasoned that bacterial dependence upon host sphingolipids could explain the decrease in *Wigglesworthia* numbers we observed in progeny of siaSMase1 knockdown mothers. Levels of *Wigglesworthia biof* were measured in the female milk gland and bacteriome organs as well as in the developing larva. Expression levels of *biof* were nearly 8-fold higher in the adult bacteriome than in the milk gland and larva (Fig. 6B). These results suggest that BioF likely plays a role in *Wigglesworthia* biotin synthesis in the adult bacteriome rather than in utilization of sphingolipids present in the milk gland or larvae. Our study shows that symbiont transfer effectiveness was

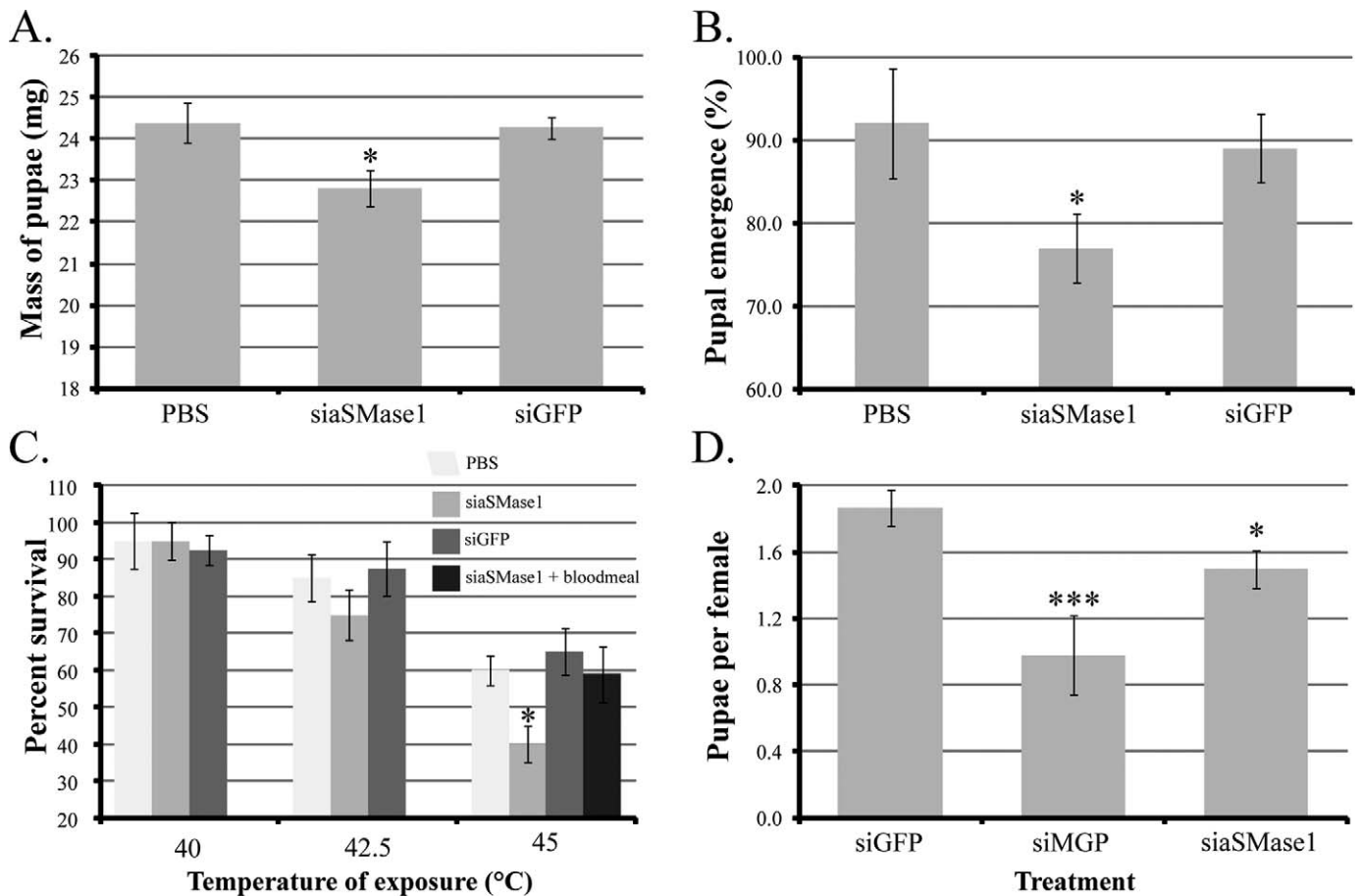


FIG. 5. Phenotypes of tsetse progeny after aSMase1 knockdown in their mother. **A)** Dry mass of pupae 5 days after deposition, mean \pm SEM of three groups of 10 pupae. **B)** Pupal hatch rate of progeny from treated mothers, mean \pm SEM of three groups of 15 pupae. **C)** Heat tolerance of teneral female flies, mean \pm SEM of three groups of 10 flies. Control flies were injected with siGFP (1 μ g/ μ l) and PBS (1 μ l). **D)** Pupal production per female over two gonotrophic cycles, mean \pm SEM of three groups of 15 individuals. A group of adult females was injected with siMGP for comparison. In the case of heat tolerance, an additional control group was sham injected. *Significant difference from siGFP-injected control following ANOVA with Tukey test ($P < 0.01$). ***Statistically lower than siGFP and siaSMase1 treatments ($P < 0.01$).

reduced by aSMase1 knockdown in the mother, which in turn could compromise the health of the progeny leading to the negative indicators we observed.

DISCUSSION

This study demonstrates that SMase is an important component of the tsetse milk secretions and is critical for success of pregnancy and subsequent health of her progeny. Our results show that *asmase1* transcripts increase nearly 1000-fold during pregnancy and that *asmase1* is the only SMase upregulated during pregnancy. Acid SMase1 is secreted, is transported to the intrauterine larva as a component of the milk, and is activated by the low pH within the larval gut to utilize milk-associated sphingomyelins. In addition, *cdase* expression in the larval gut indicates that ceramide generated by aSMase1 activity is metabolized to sphingosine and free fatty acid for absorption. Based on these results, aSMase1 activity is required for larval utilization of essential sphingolipids and their byproducts in tsetse. Furthermore, aSMase1 activity is also important for the tsetse's microbiome, which is transmitted in the milk to juvenile progeny. We have developed a model for aSMase1 synthesis, secretion, and function during pregnancy (Fig. 7). This system likely provides the majority of sphingosine derivatives required for progeny development

because milk secretions are the sole nutrient source during intrauterine larval growth and pupal development.

Insect sphingomyelin metabolism is an understudied field of research. The only other studies on insect sphingomyelin metabolism are in *Drosophila* and consist primarily of transcript localization studies [3]. Expression of SMase transcripts varies significantly between tissues and throughout development in *Drosophila* based on data provided by FlyBase [41]. Similar to *Drosophila*, tsetse has four aSMases and one nSMase, indicating these genes are conserved between these two flies. Comparison of *Drosophila cg3376* (*asmase1* ortholog) to the pregnancy-specific tsetse *asmase1* indicates that both have relatively low levels in all tissues with the exception of *asmase1* in tsetse accessory gland (milk gland) and *cg3376* in the larval fat body of *Drosophila*. Currently it is not known if *cg3376* or any other *smase* gene is expressed in *Drosophila* female accessory gland (paraovaria), because of the small size of these glands in *Drosophila* and the difficulty of their removal from the surrounding fat body. Future studies will be useful to determine if *cg3376* is expressed similarly to tsetse *asmase1* in *Drosophila* paraovaria during egg production and oviposition or if the evolutionary changes associated with tsetse viviparous reproductive physiology are responsible for the pregnancy-specific nature of tsetse *asmase1*.

Based on our knockdown studies, aSMase1 deficiency in the milk resulted in the extension of intrauterine larval

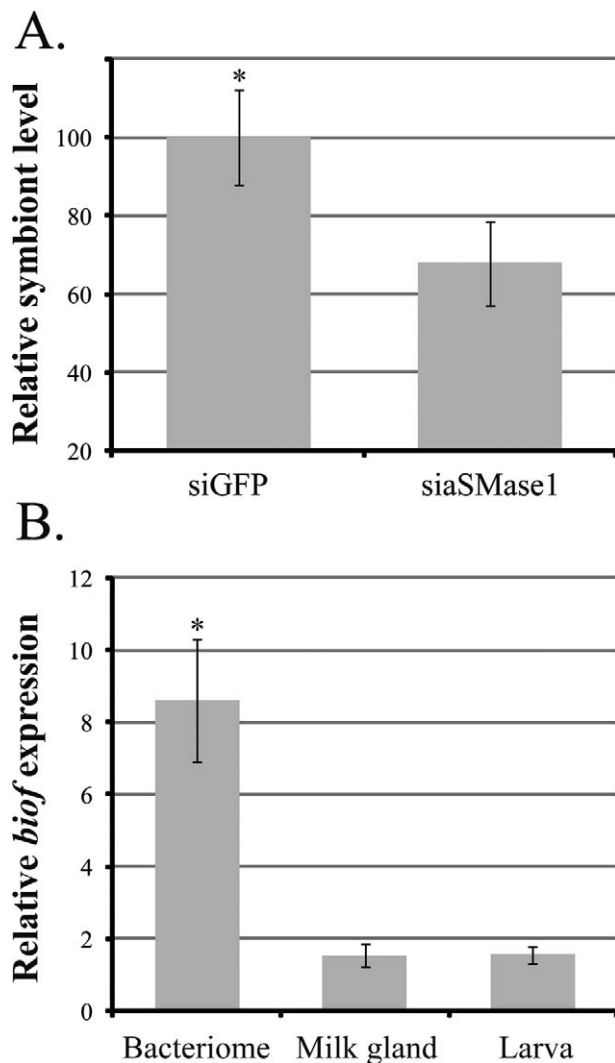


FIG. 6. Levels of obligate symbiont, *Wigglesworthia glossinidia*, and *W. glossinidia biof* in pregnant flies and specific tissues. **A)** *Wigglesworthia glossinidia* density based on *groEL* in teneral female flies in relation to tsetse tubulin, mean \pm SEM of six flies. **B)** Levels of *biof* expression, a protein with potential SPT activity, in *W. glossinidia* analyzed from tsetse fly bacteriome, milk gland, and larvae. Expression was normalized in relation to *Wigglesworthia groEL*. Data are presented as mean \pm SEM of four measurements. *Significant difference determined by ANOVA followed by Tukey test ($P < 0.01$).

development and reduced performance of emerging progeny. The aSMase1 knockdown females gave birth to slightly smaller progeny with a lower rate of pupal eclosion. In addition, emerging adult offspring displayed reduced heat tolerance. The products of sphingomyelin, sphingosine and ceramide, are associated with apoptosis, cell growth/proliferation, and metabolic processes [2, 3]. Thus, reduction of aSMase1 could impair development and contribute to the reduced pupal hatch rates. Additionally, changes in sphingolipid availability may alter cell membrane composition and signaling properties and could explain the reduced heat tolerance observed in emerging progeny from aSMase1 knockdown mothers [42, 43]. The reduced heat tolerance phenotype of the progeny was rescued following a blood meal when nutrients are made available to adult flies, given that blood is an excellent source of sphingomyelin [44]. Additionally, female progeny from SMase knockdown mothers had lower fecundity and extended pregnancy when compared to control flies. This indicates that

there are prolonged negative consequences for the progeny when juveniles do not receive adequate levels of SMase in the milk.

We also observed a reduction in the levels of *Wigglesworthia* in progeny produced by mothers with suppressed levels of *asmase1*. Reduction in *Wigglesworthia* levels is detrimental to tsetse fly physiology, as this bacterium is critical for vitamin production, reproductive fitness, and tsetse immune priming [22, 45]. It appears that proper sphingomyelin metabolism during pregnancy is important for the maintenance and/or establishment of *Wigglesworthia* populations in the larval progeny. The exact cause for reduced levels of *Wigglesworthia* following aSMase1 knockdown remains to be studied, but could result from inadequate sphingosine levels in the larval gut, which can inhibit bacterial proliferation or colonization. In *Bacteroides*, which is part of the human intestinal microflora, external sources of sphingolipids are incorporated into the bacterial cell membrane [4]. Regardless of the exact mechanism, reduced transfer of *Wigglesworthia* through the milk could account (at least partially) for the reduced performance of progeny produced by aSMase1 knockdown mothers. We found that the potential SPT ortholog, *biof*, has been retained in the small genome of *Wigglesworthia*, but orthologs for the two other genes involved in sphingolipid metabolism, 3-ketosphinganine reductase and ceramide synthase, are absent [21]. However, several bacteria known to utilize sphingolipids have been shown to lack functional orthologs of 3-ketosphinganine reductase and ceramide synthase, indicating that their absence may not be indicative of *Wigglesworthia*'s inability to process sphingolipids. Specific bacteria have been documented to utilize SMase, such as *Staphylococcus aureus* [46], but similar to *Bacteroides* and most other bacteria [4], *Wigglesworthia* lacks a distinct SMase ortholog [4]. It is possible that bacteria may use pathways different than those that occur in eukaryotes to process sphingolipids [4]. We investigated whether BioF could function in sphingolipid metabolism. Given the high levels of sphingolipids and their derivatives in the milk gland and larval gut contents, we predicted that expression of *Wigglesworthia biof* would be higher in the milk gland and larva. It was, however, significantly higher in the midgut bacteriome organ, which houses the intracellular form of *Wigglesworthia*. The function of BioF within the bacteriome is likely for biotin synthesis [21]. Even though these findings suggest that *Wigglesworthia* may not metabolize sphingolipids, the abundance of sphingolipids and sphingolipid products in the milk may lead to their incorporation into cell membrane. In *Bacteroides*, as much as 10% of the total lipid contents were documented to be ceramide even though the proper metabolic pathways for sphingolipid processing this species have yet to be identified [4, 5]. Thus, direct analysis of *Wigglesworthia* cell membranes will be necessary to determine if sphingolipids are incorporated into the cell membrane by this symbiont.

Sphingomyelins are one of the most abundant phospholipids present in mammalian milk [47, 48]. In human milk, aSMase activity is responsible for the hydrolysis of half of the milk's sphingomyelin [31]. After the initial breakdown of sphingomyelin by the SMase present within mammalian milk, the ceramide product can be metabolized by a bile salt-stimulated lipase (also present in the milk) [31]. Sphingomyelin digestion occurs in the mammalian intestinal tract by endogenous alkaline SMase and CDase activities as shown in humans and mice [7, 32, 49, 50]. Regardless, sphingomyelin in tsetse or mammalian milk requires the presence of SMase activity that is specific to and active at the proper physiological pH of the gut/intestine for the initial breakdown of sphingomyelin to PE/PC and ceramide. The co-occurrence of a CDase or CDase-like

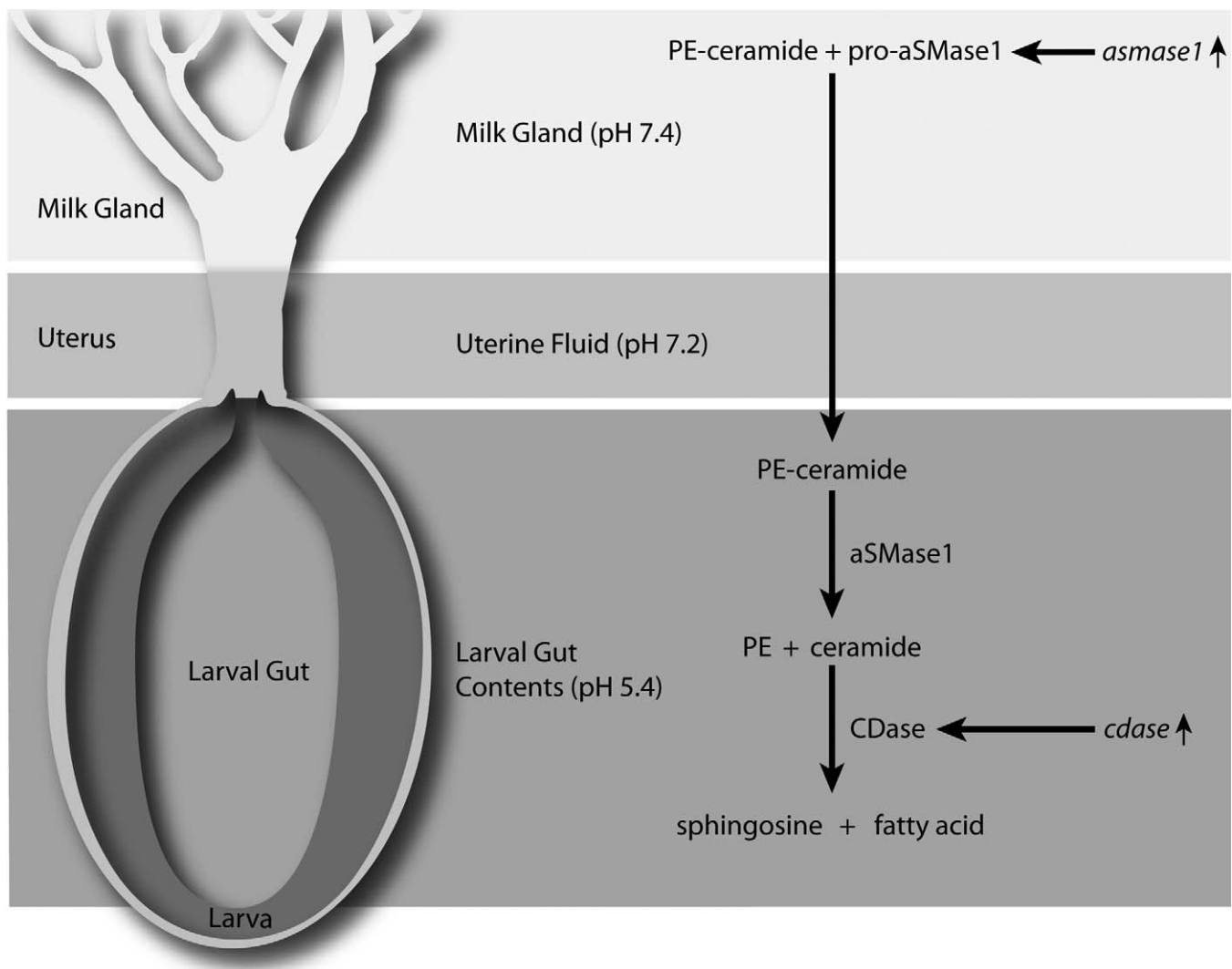


FIG. 7. Model for the role of aSMase1 during tsetse lactation and larval feeding.

activity to metabolize ceramide to sphingosine and free fatty acid for proper digestive absorption is also a functional orthology between mammals and tsetse flies [this study, 7, 31, 32]. The similarities between the mammalian and tsetse systems suggest that the presence of SMase and CDase either in the milk or within the digestive tract of the nursing juvenile is a factor critical to species that rely on the mother's milk as the sole source of nutrients during early development to obtain the sphingosine-containing molecules.

The analogies between tsetse and mammal milk production, including development of specialized lactation-associated cells, functionally conserved milk proteins, comparable lipid content of the milk, and transfer of microbial symbionts in the milk, suggests that convergent mechanisms may operate in organisms that utilize lactation as the sole source of nutrients for their developing offspring [19, 23–26]. The critical nature of SMase activity during lactation and milk consumption is an additional similarity between tsetse and mammal lactation. Functional studies with SMase in tsetse could provide information on the role of sphingomyelin digestion, metabolism, and utilization during early progeny development in lactating animals. Lastly, reduced fecundity in progeny after inadequate SMase transfer from mother to progeny indicates long-term negative consequences. Given the ease of insect cultivation, shorter

generation times, and the availability of gene silencing methodologies, the tsetse system can be readily used to understand the mechanisms underlying transgenerational effects that are more complicated to tease out in the mammalian systems.

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