Published in final edited form as:
Insect Biochem Mol Biol. 2012 May ; 42(5): 360–370.

Analysis of lipolysis underlying lactation in the tsetse fly, *Glossina morsitans*

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Abstract

Female tsetse flies undergo viviparous reproduction, generating one larva each gonotrophic cycle. Larval nourishment is provided by the mother in the form of milk secretions. The milk consists mostly of lipids during early larval development and shifts to a balanced combination of protein and lipids in the late larval instars. Provisioning of adequate lipids to the accessory gland is an indispensable process for tsetse fecundity. This work investigates the roles of Brummer lipase (Bmm) and the adipokinetic hormone (AKH)/adipokinetic hormone receptor (AKHR) systems on lipid metabolism and mobilization during lactation in tsetse. The contributions of each system were investigated by a knockdown approach utilizing *siRNA* injections. Starvation experiments revealed that silencing of either system results in prolonged female lifespan. Simultaneous suppression of *bmm* and *akhr* prolonged survival further than either individual knockdown. Knockdown of *akhr* and *bmm* transcript levels resulted in high levels of whole body lipids at death, indicating an inability to utilize lipid reserves during starvation. Silencing of *bmm* resulted in delayed oocyte development. Respective reductions in fecundity of 20 and 50% were observed upon knockdown of *akhr* and *bmm*, while simultaneous knockdown of both genes resulted in 80% reduction of larval production. Omission of one blood meal during larvigenesis (nutritional stress) after simultaneous knockdown led to almost complete supression of larval production. This phenotype likely results from tsetse's inability to utilize lipid reserves as loss of both lipolysis systems leads to accumulation and retention of stored lipids during pregnancy. This shows that both Bmm lipolysis and AKH/AKHR signaling are critical for lipolysis required for milk production during tsetse pregnancy, and identifies the underlying mechanisms of lipid metabolism critical to tsetse lactation. The similarities in the lipid metabolic pathways and other aspects of milk production between tsetse and mammals indicate that this fly could be used as a novel model for lactation research.

Keywords

adipokinetic hormone; *Glossina*; lipolysis; lactation; Brummer lipase; viviparity

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Author contributions: G.M.A, S.A., J.B.B. and L.R. designed research; G.M.A., J.B.B., G.Y., J.B., and L.R. performed research; G.M.A., J.B.B. and L.R. analyzed the research; J.B.B., G.M.A., P.T., and S.A. wrote the paper.

1. Introduction

Tsetse flies (Diptera: Glossinidae) are detrimental to human health and economic development throughout Africa as they are the sole vector of African trypanosomes, the etiological agents of sleeping sickness in humans and nagana in animals. Tsetse population control is an effective strategy to reduce trypanosome transmission due to tsetse's small population sizes and low reproductive rate. Tsetse reproduction is unique relative to other Diptera. Most dipterans are oviparous and produce large numbers of eggs each gonotrophic cycle. Tsetse undergo obligate viviparity, meaning that the mother develops a single oocyte per gonotrophic cycle, then carries and nourishes the resulting offspring in an intrauterine environment for the duration of embryonic and larval development. A critical maternal process during viviparous reproduction in tsetse is the production of a milk-like secretion produced by modified female accessory glands (milk glands) to nourish the intrauterine larvae (Ma et al., 1975). The primary component of milk during early larval development is lipids followed by a shift to a balanced mixture of protein and lipids during late larval development (Langley and Bursell, 1980; Moloo, 1976). Lactation in tsetse evolved independently from lactation in mammals, however multiple essential requirements inherent to the lactation process are conserved. 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 microbes from mother to progeny via milk secretions (Buxton, 1955; Denlinger and Ma, 1974; Denlinger and Ma, 1975; Lara-Villoslada et al., 2007; Martin et al., 2003; Moloo, 1971b).

Several, studies have focused on tsetse milk proteins (Attardo et al., 2006a; Attardo et al., 2008; Attardo et al., 2010; Attardo et al., 2006b) and there are basic studies on lipid transfer to the larva (Langley et al., 1981; Pimley and Langley, 1981, 1982). However, little is known about the regulation of lipolytic pathways in tsetse, particularly during lactation. Lipolysis of stored lipids is well-studied in *Drosophila* and is comparable to vertebrate lipolysis systems (Arrese and Soulages, 2010; Canavoso et al., 2001; Kuhnlein, 2010). One major difference from vertebrates is that there are two lipolytic systems in insects [AKH/AKHR signaling dependent lipolysis and Brummer (Bmm) lipase lipolysis] which metabolize triacylglycerides (TAG) and diacylglycerides (DAG). Vertebrates use a single lipolytic system that involves adipose triglyceride lipase (ATGL; orthologous to the Bmm in invertebrates) (Gronke et al., 2005; Gronke et al., 2007; Staubli et al., 2002). The AKH peptide hormone belongs to a class of structurally-related neuropeptides that interact with G protein-coupled receptors (GPCRs) and has functional similarities to the mammalian gonadotropin-releasing hormone, GnRH (Staubli et al., 2002). AKH is synthesized by the corpora cardiaca and is stored within secretory vacuoles until signaled to release. Secretion of AKH into the hemolymph stimulates lipolysis of triacylgylcerols (TAG) to diacylglycerols (DAG), fat body based conversion of glycogen into trehalose, and the generation of proline from alanine and lipids in some insects. This is usally a response to acute periods of starvation or increased metabolic demand (Bursell, 1977; Gade and Auerswald, 2003; Gade et al., 1997; Pimley and Langley, 1982; Van der Horst, 2003).

AKH/AKHR-dependent lipolysis operates to ensure rapid fat and nutrient mobilization. Studies in *Drosophila* show that ablation of AKH producing cells within the corpora cardiaca results in decreased levels of trehalose in the hemolymph, altered ability to regulate glucose concentrations, starvation-associated activity changes, and prolonged survival due to low levels of energy utilization (Isabel et al., 2005; Kim and Rulifson, 2004; Lee and Park, 2004). The Perilipin 1/Lipid storage droplet 1 protein (PLIN1/LSD1) is a downstream

effector of AKH (Arrese et al., 2008; Beller et al., 2010a), and is regulated by phosphorylation by Protein Kinase A (PKA). Phosphorylation of PLIN1/LSD1 increases the rate of lipolysis by promoting association with TG lipase which results in localization of lipase activity to stored fat droplets (Arrese et al., 2010; Beller et al., 2010a). Although the role of AKH during lactation in tsetse flies was unknown, extracts from the corpora cardica (the site of AKH synthesis and secretion) inhibited fat body lipid storage suggesting that AKH may play a role in the regulation of tsetse lipid metabolism (Langley et al., 1981; Pimley and Langley, 1981). Previous work has identified two neuropeptide genes in the AKH family from the tsetse fly genome, hypertrehalosaemic hormone (Phote-HrTH) and Glomo-AKH (Kaufmann et al., 2009).

Lipolysis by Bmm is an alternative to AKH mediated lipolysis and is under an independent regulatory system (Gronke et al., 2007; Zimmermann et al., 2004). In mice, humans and flies knockdown of ATGL/Bmm results in impaired lipid mobilization and an obese phenotype (Gronke et al., 2005; Gronke et al., 2007; Haemmerle et al., 2006; Zimmermann et al., 2004). Regulation of Bmm-mediated lipolysis has not yet been elucidated in insects (Beller et al., 2010a; Gronke et al., 2007; Kuhnlein, 2010). In vertebrates, ATGL lipolysis is activated by PKA-dependent phosphorylation following β-adrenergic signaling (Granneman et al., 2007; Lass et al., 2006; Wang et al., 2008; Yamaguchi et al., 2007), similar to AKH-induced lipolysis in insects (Beller et al., 2010b; Gronke et al., 2007). Phosphorylation of the mammalian comparative gene identity-58 (CGI-58) protein allows CGI-58/ATGL interaction and then translocation to the surface of lipid droplets (Granneman et al., 2007; Lass et al., 2006; Wang et al., 2008; Yamaguchi et al., 2007). Recent work in *Drosophila* shows that ceramide synthase (encoded by the *schlank* gene) down regulates the transcription of *bmm*, however, this interaction is not direct (Bauer et al., 2009). The forkhead transcription factor (FOXO) has been demonstrated to increase the Bmm-mediated lipolysis during periods of starvation in *Drosophila*, indicating that Bmm is upregulated in the absence of insulin (Wang et al., 2011). Bmm-mediated lipolysis in *Bombyx mori* shows increases in correlation with levels of 20-hydroxyecdysone. However, this is also probably an indirect effect resulting from starvation in wandering larvae prior to undergoing pupation (Wang et al., 2010).

Dual knockout of the *akhr* and *bmm* genes in *Drosophila* yields flies that are obese and that are not starvation tolerant (Gronke et al., 2007). These pathways are partially redundant as *bmm* expression increases in AKHR mutant flies or in flies lacking AKH-producing neuroendocine cells. Overstimulation from ectopic AKH expression results in reduced *bmm* expression suggesting crosstalk between systems (Gronke et al., 2007). Although these two systems have redundant functions, knockdown of one or the other results in an obese phenotype under both normal and starved conditions (Gronke et al., 2007). Thus, both AKH/AKHR and Brummer lipase lipolytic systems play important roles in the maintenance of lipid homeostasis in insects.

In this study, we examined the role(s) of the AKH stimulated and Bmm-mediated lipid metabolism in tsetse, with an emphasis on their function during lactation in pregnant flies. To do so, we characterized the expression of Bmm and AKH/AKHR system components under starvation conditions and throughout tsetse pregnancy. The physiological role of both systems was evaluated following either individual or combined knockdown of *bmm* and *akhr*. Our results show that Bmm- and AKH/AKHR-mediated lipolysis pathways are both important for lipid provisioning during milk production and thereby facilitate intrauterine larval development. The potential role of tsetse pregnancy as a model for mammalian lactation biology is discussed.

2. Materials and Methods

2.1 Flies and experimental materials

Colonies of *Glossina morsitans morsitans* at Yale University and the Institute of Zoology SAS originated from the same population of flies originally collected in Zimbabwe. Flies are maintained at 24°C with 50–60% relative humidity (RH) and receive bovine blood meals by an artificial feeding system every 48h (Moloo, 1971a). Mated female flies were collected for qPCR and western blotting according to developmental markers established in previous studies (Attardo et al., 2006a; Yang et al., 2010).

Synthetic GmmHrTH (pE-LTFSPDW-amide) was produced by New England Peptide based on sequences acquired from the tsetse fly genome. This peptide was utilized to allow comparison to the *Drosophila* AKH system, which only has a single AKH gene coding for the HrTH peptide. Antisera for GmmAKHR were prepared commercially (New England Peptide,) against a peptide generated from the first 15 amino acids of the protein based upon its antigenicity and localization to the cell surface (H_2N-MSETEVNGKIYDHRVC-OH). AKH antiserum (gift of J. A. Veenstra, Bordeaux, France) was generated against the N-terminus of locust AKH (Schooneveld et al., 1986). Tsetse Tubulin (GmmTub) antisera were developed as previously described (Attardo et al., 2006a; Guz et al., 2007).

2.2 gmmakh, gmmhrth, gmmakhr and gmmbmm gene expression analysis

Levels of *gmmakh*, *gmmhrth*, *gmmakhr*, and *gmmbmm* were determined by qPCR utilizing the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules). The data were analyzed with software version 3.1 (Bio-Rad). The primers utilized for *gmmakh*: forward 5′-TTTTCACCGGGTTGGGGTAAACG-3′ and reverse 5′-TGTTTTACAATTGCCCGTTTGTGCATC-3′, *gmmhrth*: forward 5′-GCAATTGCTTTCTTCGCTTT-3′ and reverse 5′-CGGGTTGTGTATCGAATGTG-3′, *gmmakhr*: forward 5′-TGTGCCATGTATGCCTGTCCTT-3′ and reverse 5′-ACGTTTACTTTCCCGATAGATTTTCAA-3′, and *gmmbmm*: forward 5′-GGCGGGCAGTGATAATAAT-3′ and reverse 5′-GAATGCAAGAAGCACAGAAAAG-3′. All qPCR assays were carried out in triplicate, and normalized according to *tubulin* (*gmmtub*) expression (forward 5′-CCATTCCCACGTCTTCACTT-3′ and reverse 5′-GACCATGACGTGGATCACAG-3′).

2.3 Lipid content determination

A standard vanillin assay was utilized to determine the amount of total lipids present within tsetse samples throughout the study (Benoit et al., 2010; Van Handel, 1985). Flies were removed and dried at 0% RH (relative humidity) and 60°C. Individual flies were homogenized in 2 ml of chloroform:methanol (2:1). The supernatant was removed into a 5 ml glass tube and the solvent was evaporated at 90°C. The lipids were heated in 0.4 ml of concentrated sulfuric acid at 90°C for 10 min. This is followed by adding 40 μl of the acid/lipid mixture to 4 ml vanillin reagent (Van Handel, 1985). Samples were measured spectrophotometrically at 525 nm, and total lipid content was calculated against a lipid standard (canola oil) (Van Handel, 1985).

2.4 Protein localization and expression

Protein was isolated from flash frozen female flies utilizing a modified Trizol based protocol (Park et al., 2006). Samples from pregnant flies were obtained throughout larval development and from multiple tissues for analysis. Equal volumes of protein from three flies were combined for each time point, and analyzed by standard western blotting protocols (Sambrook and Russell, 2001). Analysis of tsetse GmmTub was performed utilizing the protein equivalent of 1/400th of a fly per well. GmmAKHR analysis utilized

1/40th of a fly per well. Blots were blocked overnight in PBS, 3% BSA and 0.5% Tween 20 (blocking buffer). GmmTub specific antiserum was diluted 1:10,000 in blocking buffer, and GmmAKHR antiserum was diluted 1:5,000. Signals were visualized with Supersignal West Pico Substrate (Pierce, Wobrun, MA) on a Image Station 2000R (Kodak, New Haven, CT).

2.5 Immunohistochemical analysis

Immunohistochemical analysis with GmmAKHR antisera was performed by horseradish peroxidase-based staining (Vector Laboratories, Burlingame, CA). Fat body, milk gland, reproductive tract and midgut were isolated from pregnant females carrying larva. Tissues were stained as described (Attardo et al., 2006a) and AKH-like peptide staining of corpora cardiaca samples was performed as described (Roller et al., 2008).

2.6 In vitro fat body culture analysis of GmmHrTH function

Pregnant flies were immobilized on ice; fat body tissues were dissected and stored in culture media (1xPBS, 8% lipid free BSA and 40 mM alanine) for 3h at 25°C. The media was removed, and fat body tissues were incubated in fresh culture media with 1nM GmmHrTH, 10nM GmmHrTH, corpora cardiaca extract (20μl/ml 10^{-4} gland/μl) or hemolymph (20μl/ml) from a pregnant female, respectively. After 6h, quantification of the total lipid content within the media was determined as described (Van Handel, 1985) and standardized according to the dry mass of the fat body measured according to (Benoit et al., 2010). ANOVA followed by Tukey's post-hoc test was utilized for data analysis.

2.7 RNA interference of akhr and bmm

Plasmids containing *gmmakhr*, *gmmbmm* and *gfp* served for PCR amplification: *gmmakhr* T7 forward 5′-TAATACGACTCACTATAGGGAGAGAGCAGCCGACATACATC3′, *gmmakhr* T7 reverse 5′-TAATACGACTCACTATAGGGAGAAGGCATCAAACATAACACAC-3′, *gmmbmm* T7 forward 5′ TAATACGACTCACTATAGGGAGAGGTCTGGGTGGGAATAGT-3′, *gmmbmm* T7 reverse T7 5′-TAATACGACTCACTATAGGGAGAGTCCGATTTTTTTCGCGT-3′, *gfp* T7 forward 5′-TAATACGACTCACTATAGGGTCAGTGGAGAGGGTGAAG-3′, *gfp* T7 reverse 5′-TAATACGACTCACTATAGGCTAGTTGAACGGATCCATC-3′. Primers contain the T7 promoter sequence at the 5′ end. The PCR conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 50°C for 30 s and 72°C for 45 s and by 1 cycle at 72°C for 10 min in a MJ Research (PTC-200) thermal cycler. Purification of the PCR products was accomplished with a QIAquick PCR purification kit (Qiagen, Valencia, CA). Sense and antisense RNA were synthesized using the MEGAscript RNAi Kit (Ambion, Austin, TX), purified using RNeasy Mini Kit (Qiagen, Valencia, CA), and suspended in RNase-free water. Block-iT Dicer RNAi kit (Invitrogen, Carlsbad, CA) was used to treat the dsRNAs to improve knockdown efficiency. Concentrations were determined by spectrophotometry and adjusted to a final concentration of 200–300ng/μl. Each fly was injected with 1 μl *siRNA*. Expression levels of target genes were determined utilizing qPCR (as described above). Two groups of females containing 30 individuals each were treated with 200–300 ng *siGFP*, *siAKHR* and *siBmm*, respectively. A third group was treated with PBS within 24h post eclosion. To test the effects of double knockdown, a group of females were co-injected with *siBmm* and *siAKHR* to determine the role of suppressing both lipolytic pathways. Females were subsequently mated after 3d. Hemolymph lipids were collected by piercing the cuticle with a pulled glass capillary tube. Samples were spun at 5,000 rpm for 5 min to remove hemocytes and fat body cell contamination. Early progeny development was microscopically assessed after 8–10d and scored as: No oocyte, embryo or larvae = 0, Stage 1 oocyte (<25% yolk) = 1, Stage 2 oocyte (>25 and <75% yolk) = 2, Stage 3 oocyte (>75% yolk) = 3, intrauterine embryo = 4 and intrauterine 1st instar larvae = 5. This scoring was

used for comparison of physiological outcomes between groups. For flies monitored for pupal production over 40d, individuals were injected with *siRNA* or PBS at 10d to prolong transcript suppression. For early development studies, one group was fed 3x during the first week and in the second group the second bloodmeal (3d after emergence) was omitted to simulate a period of starvation. In experiment measuring total fecundity and gonotrophic cycle length, one group was fed 3x per week throughout the experiment and the second group was exposed to a period of starvation by omitting a single bloodmeal on 15d. This omission at 15d leads to a three day period of starvation during larvigenesis. At the end of the experiment at day 40, all flies were dissected to confirm mating status by microscopic examination of spermatheca for the presence of sperm or unovulated oocytes. Females used in the starvation experiments were not permitted to mate, and were held under colony conditions without access to blood until death.

2.8 In vivo ligation-based analysis of GmmHrTH on lipid mobilization and tsetse reproduction

The effects of GmmHrTH on whole flies and individual tissues were determined using bioassays to measure lipid levels. Pregnant flies (16–17d after emergence) were injected with 1μl of 5 μM GmmHrTH (yielding 1 nM per fly), 50 μM GmmHrTH (yielding 10 nM per fly), corpora cardiaca extract (10^{-4} glands/μl in PBS) or hemolymph from pregnant females. Changes in hemolymph and larval lipid levels were monitored over the duration of the first gonotrophic cycle by collecting hemolymph as described. Ligation at the thorax/abdomen junction was used as a physical block of GmmHrTH function on abdominal tissues including the fat body, milk gland and reproductive tract. Efficacy of the block was verified utilizing a dot blot with AKH-like peptide antisera according to (Beller et al., 2010b; Granneman et al., 2007). Following ligation, abdominal hemolymph lipid content and larval lipid content were determined as described. Results were analyzed by ANOVA analysis followed by a Tukey's post hoc test.

3. Results

3.1 Sequence alignment and phylogenetic analysis

Two tsetse AKH genes *gmmakh* (GenBank ID: HQ640947) and *gmmhrth* (GenBank ID: HQ640946) were identified in a previous study (Kaufmann et al., 2009), and the tsetse *gmmakhr* (GenBank ID: HQ640948) gene was identified from the *G. m. morsitans* genome. The mRNA sequence for *gmmakhr* was derived from a whole body tsetse cDNA library. Alignment of the *gmmakhr* predicted protein with other characterized AKHRs shows that it has significant homology to these sequences and it contains the 7 transmembrane domains characteristic of this family of proteins (Fig. S1). Phylogenetic analysis of tsetse GmmAKHR shows a similar phylogeny to that observed for GmmAKH/GmmHrTH as it is most closely related to *Drosophila* homologs (Fig. S2).

The mRNA and genomic sequences for tsetse Brummer lipase (GenBank ID: JN859183 *gmmbmm*) were identified by tBLASTn search of tsetse cDNA and genomic libraries with characterized *Drosophila* orthologs. The cDNA for *gmmbmm* is comparable to those of other insects and contains the conserved domains required for function. The conserved region contains the patatin domain that includes the catalytic regions of GASAG and GCGFLG (Fig. S3). Phylogenetic analysis of GmmBmm places it amongst higher flies (Bracycera) as is observed with GmmAKH/GmmHrTH and GmmAKHR (Fig. S4).

3.2 gmmakh, gmmhrth, gmmakhr and gmmbmm expression analysis

A time course study was performed to measure *gmmakh, gmmhrth, gmmakhr* and *gmmbmm* transcript levels in whole flies over the first two gonotrophic cycles. The stages analyzed for

gene expression are based upon the physiological status of the female reproductive tract. Mated females were dissected during the first 2 gonotrophic cycles and the pregnancy status of each female was evaluated by examining the yolk content of the developing oocyte (stages: 1 <25% yolk; 2 >25% and <75% yolk; 3 >75% yolk), the ovary in which oocyte development is occurring (left or right), the presence of an embryo or larva in the uterus and the instar of the larva in the uterus (1st instar, 2nd instar, 3rd instar).

Transcript levels of *gmmakh*, *gmmhrth* and *gmmakhr* are low early in the gonotrophic cycle (Fig. S5 a, b). Developmental stages correlating with the transition from oogenesis to larvigenesis show increased transcript levels for all three genes relative to baseline levels. During larval development, transcript levels remain at constitutive levels presumably for maintenance. For *gmmakhr*, an increase above the baseline is observed immediately after parturition (Fig. S5b). Transcript levels for *gmmbmm* increase throughout progeny development, and decline following parturition (Fig. S5c). These results suggest an early peak of transcription to build the levels of AKH-like peptides (GmmAKH/GmmHrTH) and GmmAKHR in the corpora cardiaca and fat body, respectively. This is followed by lower constitutive levels of expression for maintenance. The increased expression of *gmmbmm* during larval development may reflect the need for more mobilized lipids that are necessary to feed the developing larva as pregnancy progresses (Fig. S5c).

3.3 Protein localization of GmmAKH-like peptides and GmmAKHR and transcript localization of gmmbmm

Tissue specific western blot analyses indicate that GmmAKHR expression is specific to the fat body/milk gland and larval samples (Fig. 1a). Immunohistochemical analysis of these tissues confirms that GmmAKHR is associated with the fat body (Fig. 1b), while GmmAKH-like peptides are localized to cells of the corpora cardiaca (Fig. 1c). The spatial distribution patterns of GmmAKH/GmmHrTH-like peptides and GmmAKHR are similar to those observed in other insect systems. Transcript levels of *gmmbmm* are high in energy-storing tissues and sites of fat digestion, in particular the midgut, bacteriome and fat body/milk gland (Fig. 1d). Additionally, *bmm* is highly expressed within the developing larvae.

3.4 In vitro Fat Body Culture Analysis of HrTH Function

Fat body tissue from adult female tsetse were tested to determine if stimulation with synthetic GmmHrTH results in lipid secretion (Fig. 2). Fat body tissue incubated in media containing GmmHrTH secreted five times more lipid than in control media (Fig. 2). Addition of extracts from the corpora cardiaca and hemolymph of pregnant females also increased the amount of secreted fat body lipid by 2 to 3-fold, suggesting that these extracts contain factors promoting lipid secretion. These results indicate that tsetse fat body responds to GmmHrTH stimulation by metabolizing and secreting lipid stores.

3.5 In vivo analysis of GmmHrTH on lipid mobilization and tsetse reproduction

Hemolymph lipids increase during the first 2–3 days after adult emergence, and remain constant throughout the gonotrophic cycle at 10–13μg/μl of hemolymph (Fig. 3a). Ligation of 16–17 day old females carrying 2nd instar larvae between the thorax and abdomen resulted in decreased hemolymph lipids in the abdomen within 1h (Fig. 3b). GmmHrTH was not detectable by dot blot analysis in the ligated abdomen (Fig. S6). This phenotype was partially alleviated by ectopic Phote-HrTH treatment *via* abdominal injection (Fig. 3b). These results indicate that mechanical blockage of chemical cues from the head/thorax leads to reduced abdominal hemolymph lipid levels, and GmmHrTH is likely one of the cues that regulates these levels.

3.6 Effects of gmmakh and gmmbmm knockdown on female survival, lipid content and tsetse reproduction

siRNA treatment of flies resulted in reduction of *gmmakhr* and *gmmbmm* transcripts by at least 75% in comparison to PBS-injected and *siGFP*-injected flies (Fig. 4a). Individual knockdown of *gmmakhr* and *gmmbmm* led to reduced hemolymph lipid levels. Combined knockdown of *gmmakhr* and *gmmbmm* resulted in over a 50% reduction in the hemolymph lipid levels (Fig. 4b). No difference was noted in the hemolymph lipid levels after injection of PBS or *siGFP* (Fig. 4b). Intrauterine larval expression levels of *gmmakhr* and *gmmbmm* from mothers receiving the siRNA treatments were not significantly different between control and knockdown flies (figure not shown). Suppression of *gmmakhr* or *gmmbmm* resulted in increased expression levels of the other (Fig. S7a, b). In addition, continual injection of GmmHrTH resulted in reduced *gmmbmm* transcript levels (Fig. S8c).

Female flies under starvation conditions after either *gmmakhr* and *gmmbmm* knockdown lived nearly 40 h longer than the control flies (Fig. 5a). Simultaneous knockdown of both *gmmakhr* and *gmmbmm* extended survival by 75 h relative to controls and 30–35 h longer than those injected with either *siBmm* or *siAKHR* alone (Fig. 5a). Analysis of female lipid content at the time of death revealed that flies injected with *siAKHR* or *siBmm* had higher lipid levels than those treated with PBS or *siGFP* (Fig. 5b). Combined knockdown of *siAKHR* and *siBmm* resulted in higher lipid content at time of death than when each gene is suppressed alone (Fig. 5b). These results indicate that suppressing *gmmbmm* or *gmmakhr* leads to extended fly survival, likely due to the reduced rate of lipolysis during starvation and the inability of flies to fully utilize lipid reserve before death.

Impact of these knockdown experiments on oocyte and embryonic development was also measured. Knockdown of *gmmakhr* did not impact oocyte/early progeny development. However, knockdown of *gmmbmm* caused a significant delay in oocyte/early progeny development (Fig. 6a). The co-suppression of *gmmakhr* and *gmmbmm* led to a similar developmental delay as was observed in the *gmmbmm* suppression alone (Fig. 6a). Starvation stress (created by omission of a blood meal during pregnancy) in combination with knockdowns resulted in increased severity of the previously observed phenotype in all treatments (Fig. 6b). No difference was noted in oocyte/early progeny development in controls during continual feeding or after omission of a bloodmeal (PBS or *siGFP* injected flies, Fig. 6b). These results indicate that the GmmBmm-mediated lipolytic system is important for oocyte development under continual feeding conditions and both GmmAKH/GmmAKHR- and GmmBmm-mediated lipolytic systems are critical during starvation. The reduction of available hemolymph lipids could explain the delay in oocyte development.

Knockdown of the GmmBmm and the GmmAKH/GmmAKHR-mediated lipolytic systems affected larvigenesis as well. Control females were capable of producing approximately 1.8 offspring/fly within 40d of emergence (Fig. 6c, e). Suppression of *gmmbmm* and *gmmakhr* lowered the production to 0.8 and 1.46 offspring per female, respectively (Fig. 6c, e). Combined knockdown of *gmmbmm* and *gmmakhr* reduced offspring production to less than 0.5 offspring per female in 40d (Fig. 6c, e). Control female flies under nutritional stress induced by omission of a bloodmeal at 15d, showed increased larval development time relative to the unstressed controls shifting from 21 to 24d average larval development time (Table S1). However, the overall number of pupae per female was not significantly impaired (Fig. 6d, f). When *gmmbmm*, *gmmakhr* and *gmmbmm*/*gmmakhr* knockdowns were followed by nutritional stress at 15d, pupal production was reduced even further than that observed with gene knockdown alone (Fig. 6d, f). These results indicate that the GmmAKH/GmmAKHR- and GmmBmm-mediated lipolytic systems are each critical for maximum tsetse fecundity. The suppression of the function of both systems is extremely detrimental to tsetse reproduction as it results in 80% reduction of progeny development. The addition of

nutritional stress to these experiments almost completely disrupts the reproductive cycle, indicating that these lipolytic systems are essential for resource management during periods of nutritional stress in the wild.

Total lipid levels in females were followed throughout pregnancy after larval removal. The total lipid content within control (*siGFP*-injected) flies increases progressively until the beginning of larvigenesis (13–15d; Fig. 7), and is followed by a decline until parturition (19–21d). After parturition, the lipid content of flies increases again prior to the second gonotrophic cycle. Flies with a double knockdown of *gmmakhr* and *gmmbmm* show rapid accumulation of lipids and reach higher levels of stored lipids than control flies. During pregnancy, lipid levels in double knockdown flies do not decline as observed in control flies (Fig. 7). Based on these results, *gmmahkr* and *gmmbmm* suppression results in obese females incapable of utilizing their lipid reserves during pregnancy for milk production.

4. Discussion

We show here that the two lipolytic systems identified in *Drosophila*, the adipokinetic hormone/receptor (AKH/AKHR) system and the Brummer (Bmm) lipase system, are conserved in tsetse (Gronke et al., 2005; Gronke et al., 2007). This is the first reported demonstration of a functional role for the Bmm-mediated lipolysis in reproduction. Our results demonstrate that both the AKH/AKHR and Bmm-mediated lipolysis systems are important for maintenance of hemolymph lipid levels during pregnancy. However, Bmm-based lipolysis may play a dominant role in lipid provision during tsetse lactation.

In *Drosophila*, the AKH/AKHR system functions in response to rapid changes in lipid demands, while Bmm functions to maintain lipid levels for the metabolic baseline (Gronke et al., 2007). It is important to note that the two pathways are somewhat redundant in regards to lipid metabolism. When one of the pathways is blocked, the other appears capable of partial compensation. Of interest is that *Drosophila akhr*$^{-}$/*bmm*$^{-}$ double mutants die more rapidly than control or either *akhr*$^{-}$ or *bmm*$^{-}$ only flies under starvation conditions. In addition the starved flies died with high levels of stored lipids. This is likely due to the flies complete inability to utilize lipid stores (Gronke et al., 2007).

In *Glossina*, we show similar results after suppression of either *gmmakhr* or *gmmbmm*, however suppression of both genes results in extended survival during starvation surpassing that of the individual knockdown effects. The difference between these phenotypes is likely due to the *siRNA* based knockdown of *gmmbmm* and *gmmakhr*, rather than the genomic knockouts utilized in *Drosophila* (Gronke et al., 2007). The incomplete nature of the *siRNA*-based technique causes slower lipid utilization rather than a complete block, resulting in extended survival rather than rapid starvation. While survival rates between the two species differ after suppression of the two lipolysis systems, both *Drosophila* and *Glossina* have a similar phenotype of high remaining levels of stored lipids after death by starvation. This suggests that the AKH/AKHR and Bmm pathways fulfill similar functions within different biological contexts in *Drosophila* and *Glossina.*

In the early stages of tsetse pregnancy prior to larval development, female lipid levels increase to ~ 5 mg per fly. Over the course of intrauterine larval development, maternal lipid content plummets to ~ 2 mg. Knockdown of both *gmmbmm* and *gmmakhr* during this process resulted in a rapid increase in lipid stores that reach higher levels than that of in normal flies. However, *gmmbmm* and *gmmakhr* knockdown flies appear incapable of utilizing these lipid reserves during pregnancy resulting in delayed larval development and a severe reduction in fecundity. Oocyte development still progresses (albeit at slower rate) suggesting that pregnancy fails due to failed embryogenesis or incomplete larval development due to death and/or abortion. This supports the idea that reproductive

disruption from double knockdown of *gmmbmm* and *gmmakhr* is due to the fly's inability to metabolize stored lipids required for lactation during larvigenesis. Given the scale of lipid metabolism and mobilization required for tsetse reproduction (~ 8–10 mg of lipids are required for milk production/larval development per gonotrophic cycle), it appears however that both pathways function in progeny development.

In Holstein dairy cows, expression of *atgl* (the ortholog of *bmm*) is low during early lactation and increases as the lactation process progresses (Koltes and Spurlock, 2011). Transcriptomic studies on adipose tissue from dairy cows during lactation revealed a general trend of increased levels of lipolysis-associated gene expression and a decrease in those associated with lipogenesis (Sumner-Thomson et al., 2011). During bovine lipolysis, ATGL function appears to be regulated by post-translational control during lactation to increase the rate of lipolysis (Koltes and Spurlock, 2011; Sumner-Thomson et al., 2011). ATGL enzymatic activity is probably regulated by its phosphorylation dependent association with the CGI-58 protein, which results in its translocation to the surface of lipid storage droplets during pregnancy (Beller et al., 2010b; Kuhnlein, 2010; Wang et al., 2008; Zimmermann et al., 2004).

In pregnant tsetse, Bmm appears critical for lipolysis as its knockdown drastically suppresses fecundity. Based upon current data, we do not know if *bmm* is under transcriptional or translational control during tsetse lactation. There is little in the way of transcript variation throughout pregnancy, which suggests that regulation of *bmm* in tsetse may occur post-transcriptionally, in a similar manner to that of ATGL regulation in cows and other mammals.

The role of AKH during insect reproduction has been studied only in a few insects. In the nematode, *Caenorhabditis elegans* (Lindemans et al., 2009) and the cricket, *Gryllus bimaculatus* (Lorenz, 2003), AKH injections result in smaller ovary size due to development of fewer terminal oocytes and a reduced rate of oocyte maturation (Lorenz, 2003; Lorenz and Gade, 2009). This phenotype is a probable result of reduced fat body energy stores during vitellogenesis as AKH inhibits lipid storage. The inability to accumulate nutrient reserves after AKH exposure was demonstrated in the locust, *Schistocerca gregaria*, and the mosquito, *A. aegypti* (Gokuldas et al., 1988; Ziegler, 1997). In addition, AKH inhibited the expression of reproduction specific proteins (Carlisle and Loughton, 1979; Glinka et al., 1995; Moshitzky and Applebaum, 1990). Recent work shows that *C. elegans* AKH signaling through the gonadotropin releasing hormone receptor is involved in egg development and deposition (Lindemans et al., 2009). RNAi of CeAKH/CeGnRH and its receptor results in a delay in egg production and a reduction in the number of eggs produced (Lindemans et al., 2009).

We show that reducing AKHR levels by RNAi in tsetse results in delayed larval development under continual feeding and results in fewer progeny in tsetse. However, omission of a bloodmeal during larvigenesis after knockdown of *gmmakhr* leads to delayed oogenesis and larval development. This suggests that AKH/AKHR mediated lipolysis in tsetse reproduction is especially important for nutrient regulation during periods of nutritional stress.

Both lipolytic pathways play an important role in regulating nutrient transfer during larvigenesis in tsetse. During oogenesis and embryogenesis (when milk is not synthesized) lipolysis activity by both pathways is low or downregulated and dietary nutrients are shunted to the fat body for storage as lipids (Figure 8a). During pregnancy, stored fat body lipids are transferred to the milk gland in a dramatic shift as described in Langley and Bursell (Langley and Pimley, 1979). Maintenance of hemolymph lipid levels in response to the

demand by the milk gland during lactation requires a steady flow of lipids to the milk gland to ensure optimal intrauterine larval development. Lipids are an essential nutritional component of the milk for developing larvae and are therefore indispensable for reproduction. It appears that Bmm-based lipolysis plays a dominant role as knockdown of this system is more detrimental to larval production than *gmmakhr* knockdown (Figure 8b). However, both systems appear critical for flies to reach their maximum reproductive potential. This requirement for both pathways to maintain fecundity is apparent under conditions of nutritional stress or during late larvigenesis when lipid stores are low and imbibed blood volumes are limited by a reduced in the number of bloodmeals or the size of the intrauterine larva, respectively (Figure 8c). The combination of both systems provides the flexibility to respond to different environmental/nutritional conditions by using the strengths of one system to compensate for weakness of the other. While tsetse and mammalian lactation systems developed independently, the basic processes of lipolysis underlying the movement of lipids for milk production are comparable and an interesting example of convergent evolution.

Aspects of nutrient mobilization from female tsetse to intrauterine progeny are orthologous to mammalian lactation. The tsetse accessory gland (milk gland) is analogous in function to mammary glands. Tsetse lactation products have parallels to those of mammals (Attardo et al., 2006a; Attardo et al., 2010; Guz et al., 2007; McManaman and Neville, 2003). These parallels include similarities in the nutritional composition of the milk, functional orthology of milk proteins, and investment of substantial nutrient reserves for the developing offspring (Attardo et al., 2008; Cmelik et al., 1969; Martin et al., 2003; Tobe and Langley, 1978). In addition, tsetse's obligate symbiont (*Wigglesworthia*) is maternally transmitted to progeny via the mother's milk secretions. The bacteria colonize the bacteriome tissue within the larval gut and probably the developmental precursor to the milk gland tissue in females. In mammals, gut fauna are also transferred from mother to offspring during the ingestion of lactation products (Denlinger and Ma, 1974; Denlinger and Ma, 1975; Lara-Villoslada et al., 2007; Martin et al., 2003; Tobe and Langley, 1978).

Recently, *Drosophila* was proposed as a model system for lipolysis in humans and other vertebrates due to the evolutionary conservation in lipolytic enzymes (Arrese and Soulages, 2010; Beller et al., 2010b; Gronke et al., 2007; Kuhnlein, 2010). The presence of similar lipolytic pathways between tsetse, *Drosophila* and mammalian systems coupled with the transfer of milk that occurs during tsetse pregnancy, suggest that tsetse could represent an exceptional model system for lipid metabolic processes during mammalian lactation. In particular, the functional orthologs required for generation, regulation and transportation of lipids for lactation can be identified and manipulated to understand critical processes and regulatory pathways essential for milk production in mammals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this project was provided by NIH AI081774 and Ambrose Monell Foundation Awards to SA, NIH F32AI093023 award to JBB, and by the European Fund for Regional Development (EFRD). Grant:ITMS 26240220020 to PT. We thank Oleg Kruglov and Yineng Wu for their technical expertise.

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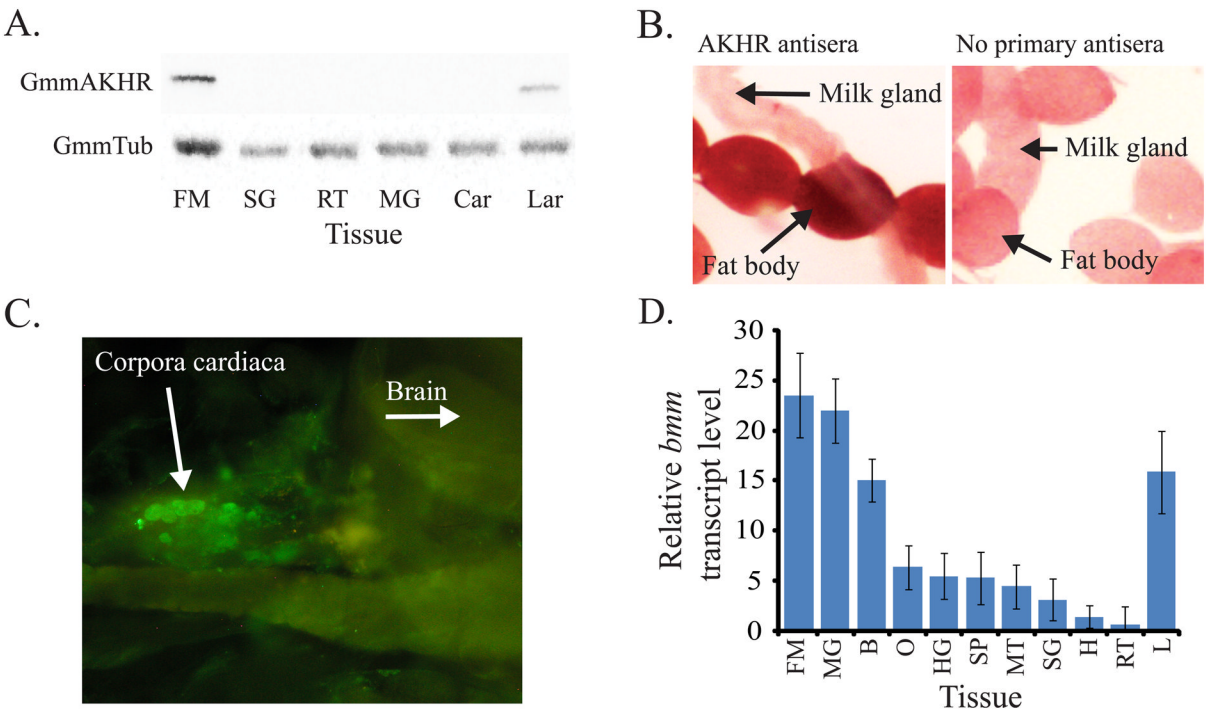

Figure 1.

Temporal and spatial analysis of GmmAKHR, GmmAKH/HrTH, and *gmmbmm* (A). Western blot localization of GmmAKHR in fat body/milk gland (FM), salivary gland (SG), reproductive tract (RT), midgut (MG), remaining carcass (Car) and larvae (Lar). (B). Localization of GmmAKHR (left panel, GmmAKHR-antisera as primary antibody, right panel, no primary antibody) (C). Localization of GmmAKH/HrTH using immunohistochemistry. (D.) Quantitative PCR of *gmmbmm* from tissues recovered from pregnant females including fat body/milk gland (FM), midgut (MG), bacteriome (B), ovaries (O), hindgut (HG), spermatheca (SP), malphigian tubules (MT), salivary glands (SG), head (H), reproductive tract (RT) and larva (L). Results are displayed as the mean ± SE of three samples, and samples are normalized to *tubulin* levels.

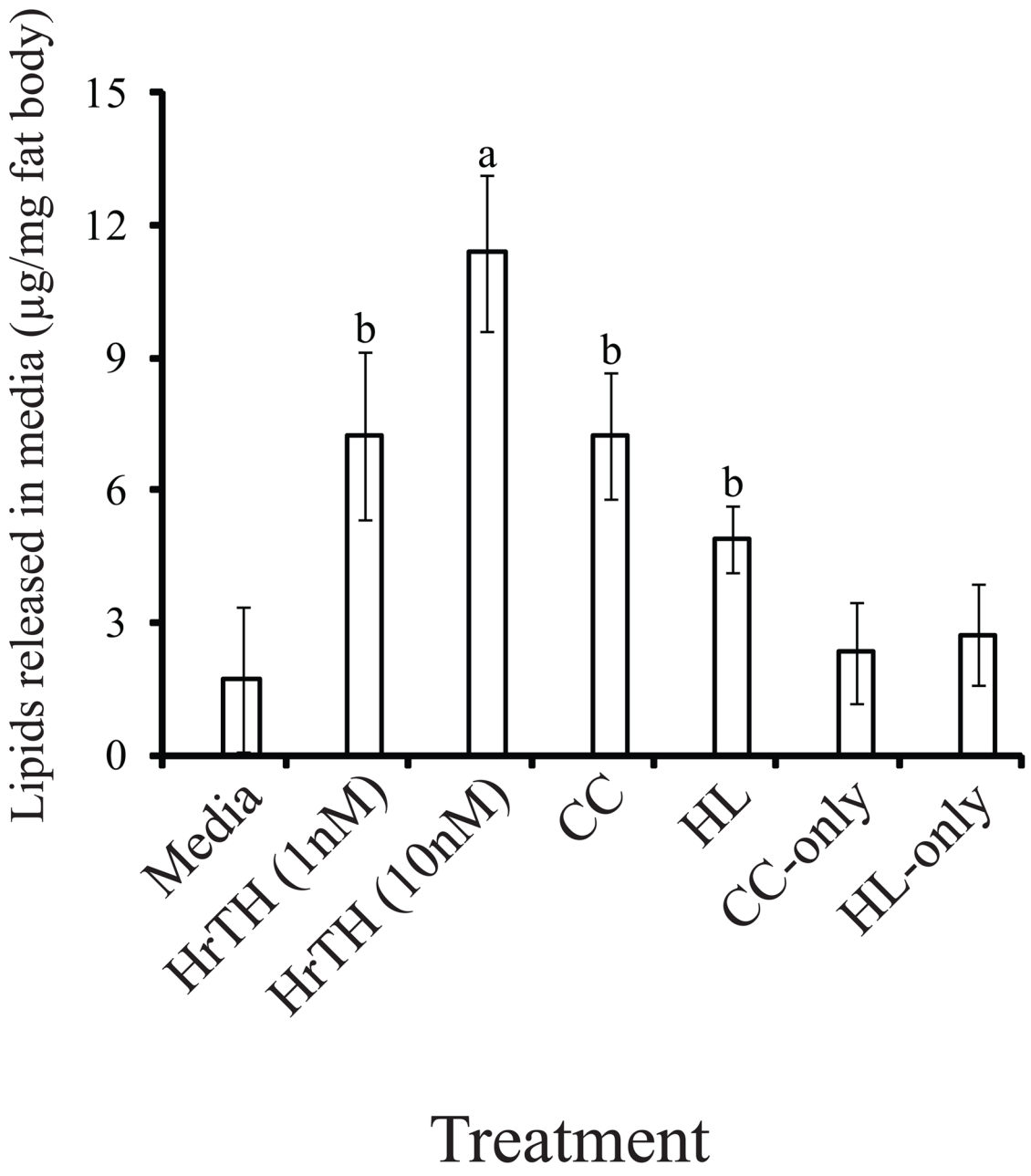

Figure 2.

Effect of synthetic HrTH on tsetse fat body cultured *in vitro*. Media, Media-only; HrTH 1nM, media supplemented with 1nM HrTH; HrTH 10nM, media supplemented with 10nM HrTH; CC, media supplemented with corpora cardiaca extracts (1 pair per 50 µl), HL, media supplemented with 5µl of hemolymph from pregnant females per ml. CC-only and HL-only, no fat body present in media. Each sample represents the mean ± SE of 15 individuals from five separate experimental groups of three. Mean values were compared using ANOVA followed by Tukey's test ($P < 0.05$). a, denotes significant difference from controls ($P < 0.05$). b, represents a significant difference than both controls and those denoted with a ($P < 0.05$).

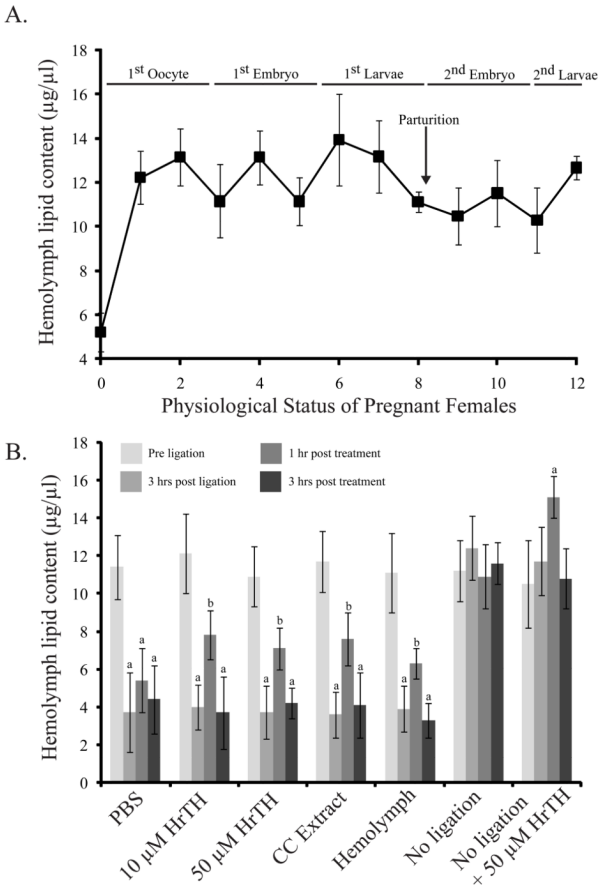

Figure 3.

Hemolymph lipid content of pregnant females. A. Lipid content in the hemolymph throughout the first and second gonotrophic cycles. B. Changes in hemolymph total lipid content after ligation. Ligation, indicates that flies were ligated at thorax/abdomen junction; 1µl 10µM HrTH injection; Each sample represents the mean ± SE of 15 individuals from five separate experimental groups of three. a, denotes significant difference from controls ($P < 0.05$). b, represents a significant difference than both controls and those denoted with a ($P < 0.05$).

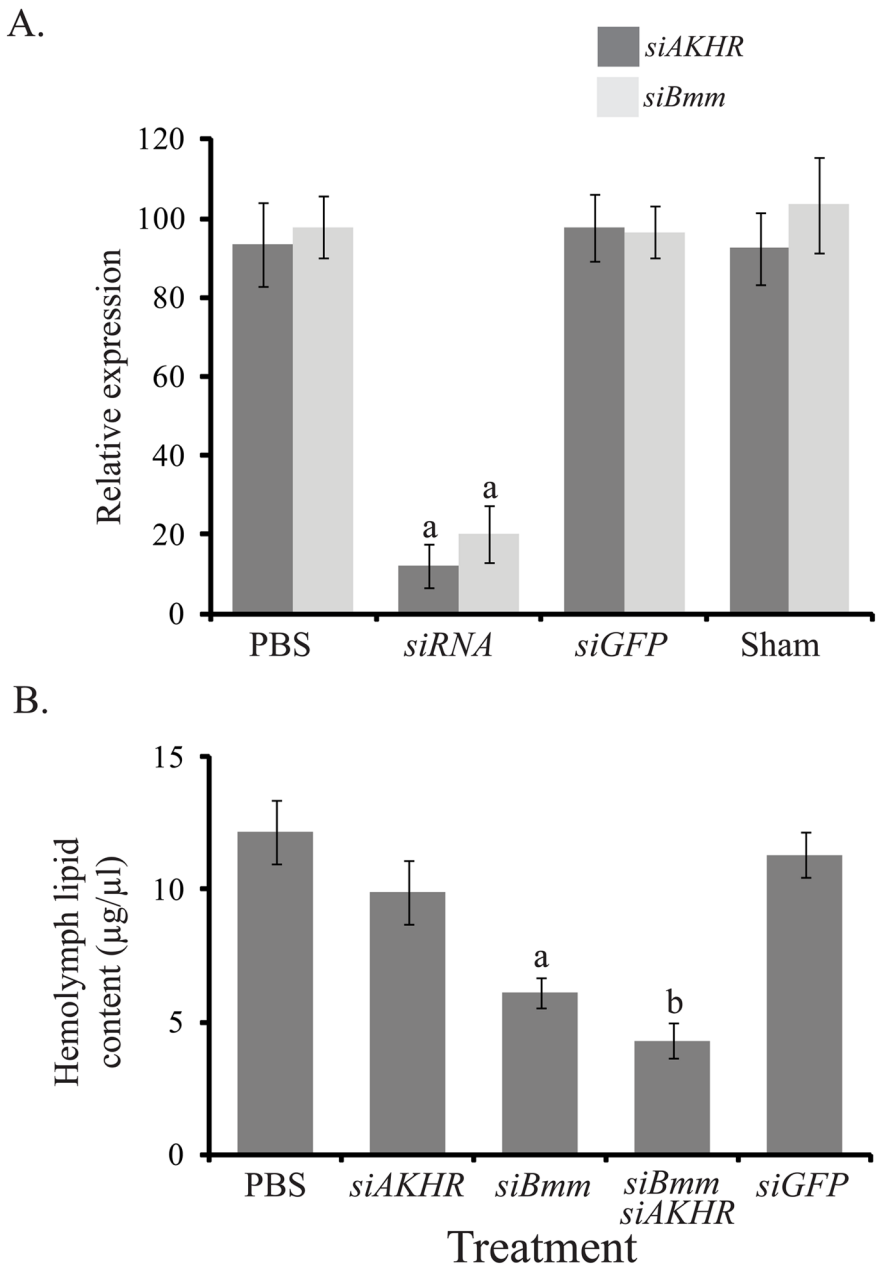

Figure 4.

Effects of *gmmbmm* and *gmmakhr* knockdown on transcript and hemolymph lipid levels. A. Target gene transcript levels after siRNA treatment as determined by qPCR (each sample represents 3 groups of 5 flies). B. Total hemolymph lipids after siRNA treatment. Each sample represents the mean ± SE of 5 individuals 3d after injection. a, represents significant difference from the control ($P < 0.05$), b, represents a significant difference than both controls and those denoted with a; $P < 0.05$).

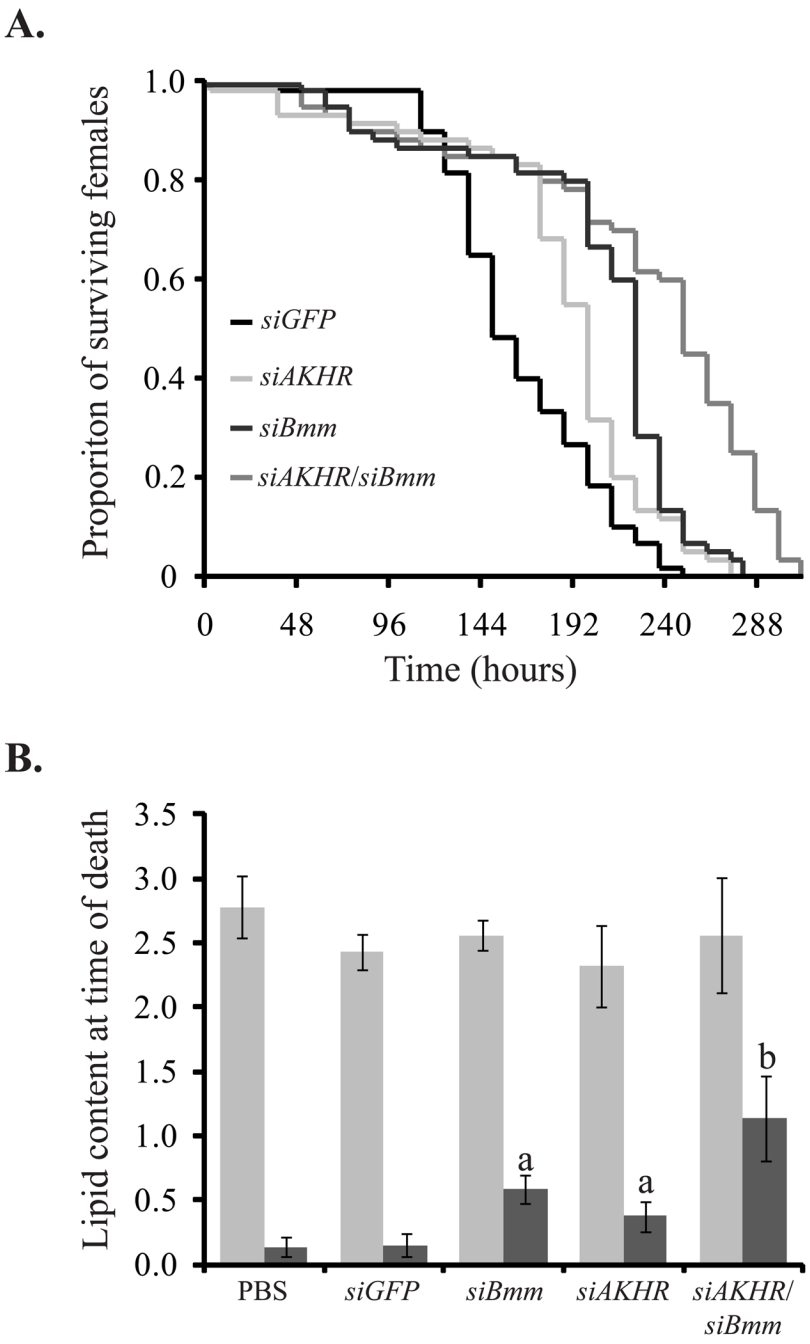

Figure 5.

Survival of unmated female flies following injection of PBS, *siGFP*, *siAKHR* and *siBmm*. (A) Survival of tsetse females after siRNA injection and under starvation conditions (PBS-injected flies are not shown since the results are not statistically different from siGFP-injected flies) (B) Lipid content at time of death. Each sample represents the mean ± SE of 20 individuals from three experimental replicates for a total of 60 flies. Survival data was analyzed with Kaplan-Meier plot with a log rank test. a, denotes significantly higher than control ($P < 0.05$); b, denotes significantly higher than individual knockdown.

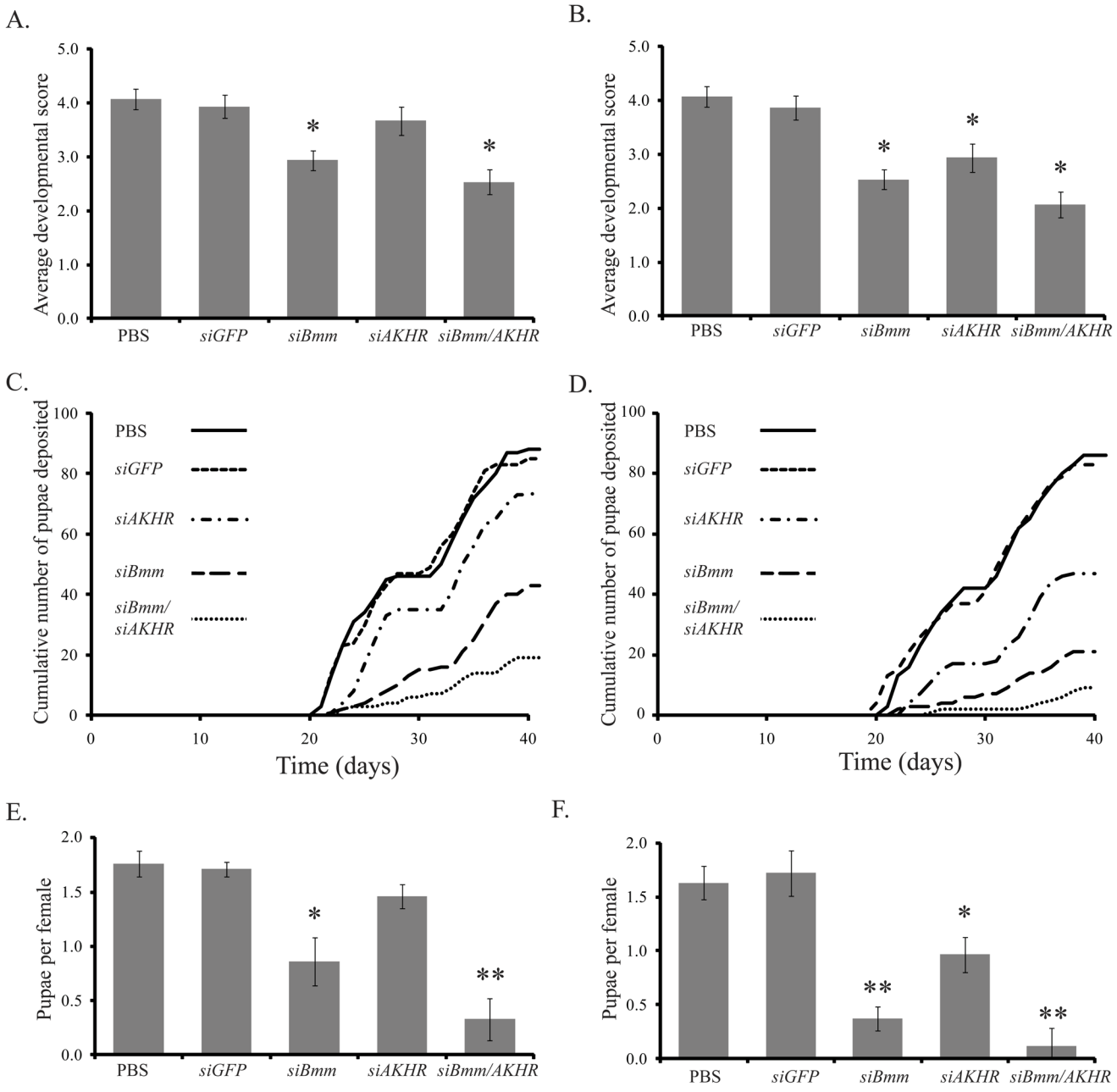

Figure 6.

Effects on progeny production with and without nutritional stress following injection of PBS, *siGFP*, *siAKHR*, *siBmm* or *siAKHR*/*siBmm* combination. (A) Oocyte development following injection of PBS, *siGFP*, *siAKHR*, *siBmm* and *siAKHR*/*siBmm* combination. Each sample represents the mean ± SE of three groups of 50 females. *, represent significant difference from controls by p-value < 0.05. Early progeny development was microscopically assessed and scored as: No oocyte, embryo or larvae = 0, Stage 1 oocyte (<25% yolk) = 1, Stage 2 oocyte (>25 and <75% yolk) = 2, Stage 3 oocyte (>75% yolk) = 3, intrauterine embryo = 4 and intrauterine 1st instar larvae = 5. (B) Early progeny development following injection of PBS, *siGFP*, *siAKHR*, *siBmm* and *siAKHR*/*siBmm* combination. Each sample represents the mean ± SE of three groups of 50 females with bloodmeal omitted on day 3. *,

*, represent significant difference from controls by p-value < 0.05.. Embryonic development was scored as described in A. (C) Effects of siRNA treatment on total progeny production. Data represents total pupal deposition by 50 females over 40d after eclosion. (D) Effects of siRNA treatment effects and nutritional stress on total progeny production. Data represents total pupal deposition by 50 females over 40d after eclosion. Nutritional stress was created by withholding a bloodmeal on day 15 post eclosion. (E) Average progeny production per female over 40d. Each sample represents the mean ± SE of three groups of 40 females. (F) Average progeny production per female over 40d with nutritional stress. Each sample represents the mean ± SE of three groups of 40 females. Nutritional stress was created by withholding a bloodmeal on day 15 post eclosion. *, indicates significantly different than controls (PBS and siGFP treated) by p-value < 0.05. **, indicates significantly different than control by p-value < 0.01,

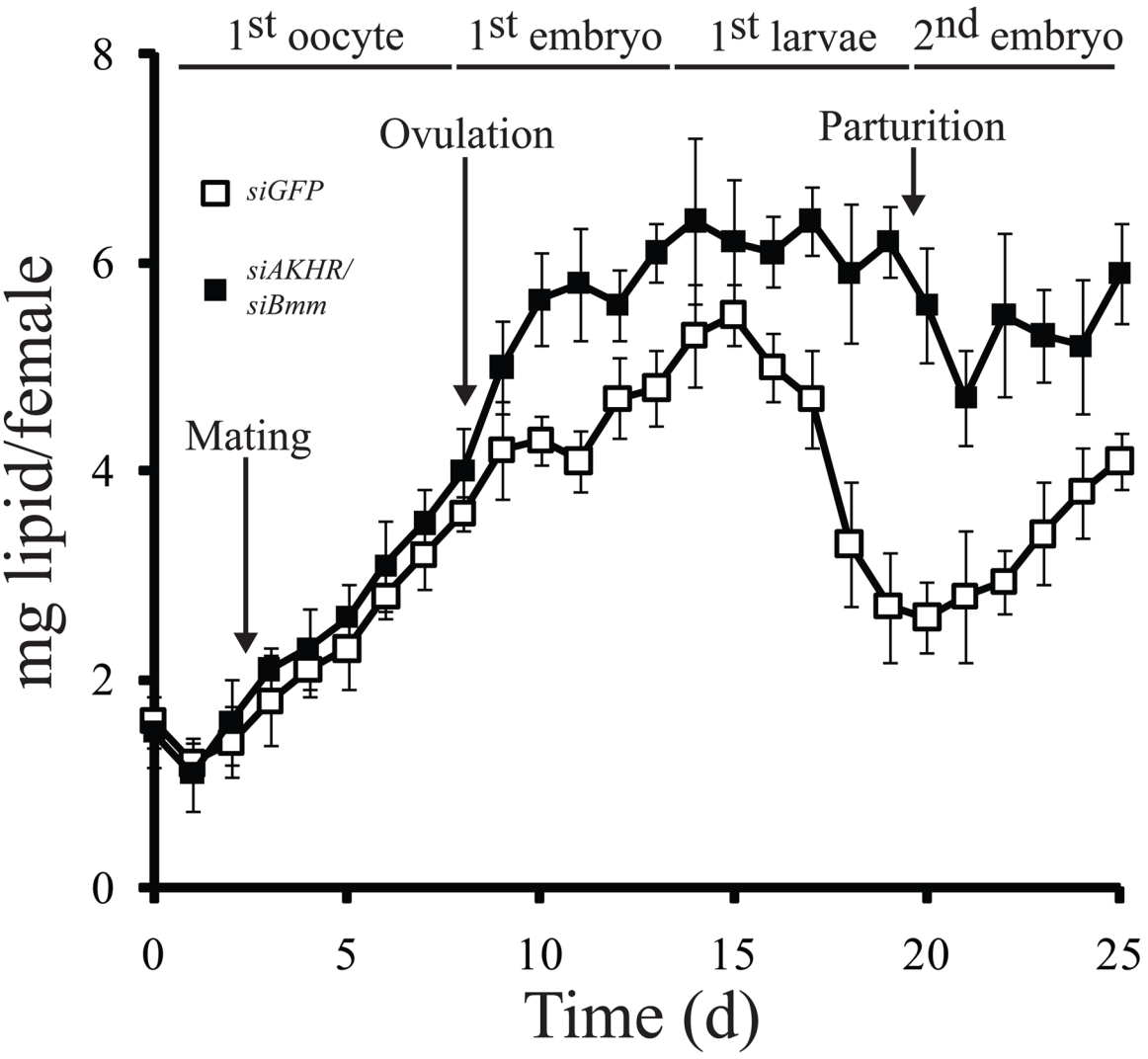

Figure 7.
Lipid content of female tsetse flies throughout pregnancy following injection of *siAKHR/siBmm* or *siGFP* (control). Each point represents the mean ± SE of two groups of 5 flies.

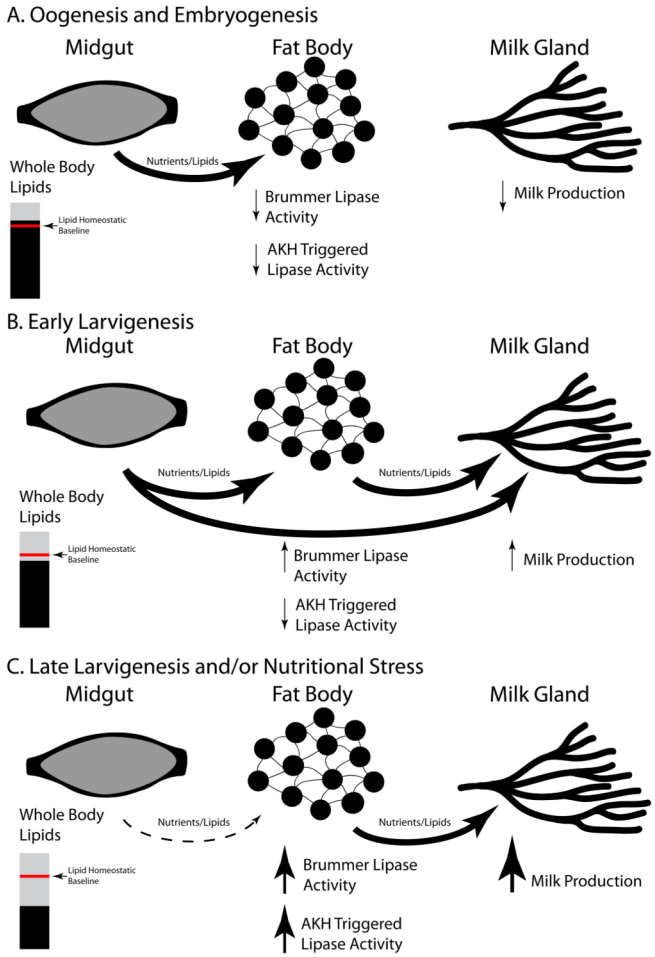

Figure 8.

Proposed model of lipolysis in female tsetse flies during different stages of reproduction. A. Oogenesis and embryogenesis, B. Early larvigenesis and C. Late larvigenesis. Lipid levels above the homeostatic baseline indicates there are ample circulating lipids to allow storage and progeny generation. Below this baseline, lipids will be mobilized from the fat body for survival during starvation of the fly and/or allocation to the production of milk.