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Lipophorin acts as a shuttle of lipids to the milk gland during tsetse fly pregnancy

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Abstract

During pregnancy in the viviparous tsetse fly, lipid mobilization is essential for the production of milk to feed the developing intrauterine larva. Lipophorin (Lp) functions as the major lipid transport protein in insects and closely-related arthropods. In this study, we assessed the role of Lp and the lipophorin receptor (LpR) in the lipid mobilization process during tsetse reproduction. We identified single gene sequences for GmmLp and GmmLpR from the genome of *Glossina morsitans morsitans*, and measured spatial and temporal expression of *gmmlp* and *gmmlpr* during the female reproductive cycle. Our results show that expression of *gmmlp* is specific to the adult fat body and larvae. In the adult female, *gmmlp* expression is constitutive. However transcript levels increase in the larva as it matures within the mother's uterus, reaching peak expression just prior to parturition. GmmLp was detected in the hemolymph of pregnant females and larvae, but not in the uterine fluid or larval gut contents ruling out the possibility of direct transfer of GmmLp from mother to offspring. Transcripts for *gmmlpr* were detected in the head, ovaries, midgut, milk gland/fat body, ovaries and developing larva. Levels of *gmmlpr* remain stable throughout the first and second gonotrophic cycles with a slight dip observed during the first gonotrophic cycle. GmmLpR was detected in multiple tissues, including the midgut, fat body, milk gland, spermatheca and head. Knockdown of *gmmlp* by RNA interference resulted in reduced hemolymph lipid levels, delayed oocyte development and extended larval gestation. Similar suppression of *gmmlpr* did not significantly reduce hemolymph lipid levels or oogenesis duration, but did extend the duration of larval development. Thus, GmmLp and GmmLpR function as the primary shuttle for lipids originating from the midgut and fat body to the ovaries and milk gland to supply resources for developing oocytes and larval nourishment, respectively. Once in the milk gland however, lipids are apparently transferred into the developing larva not by lipophorin but by another carrier lipoprotein.

Keywords

Lipid movement; lipophorin; tsetse development; *Glossina*

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1. Introduction

The reproductive process in the tsetse fly represents a drastic shift in physiology from oviparous reproduction (egg deposition) to obligate viviparity (intrauterine development and nourishment of a progeny over the duration of larval development) (Meier et al., 1999; Tobe and Langley, 1978). To accommodate this mode of reproduction, tsetse reproductive morphology has undergone drastic alterations (Tobe and Langley, 1978). The birth canal is adapted into a uterus to accommodate the developing larvae and the accessory gland (milk gland) is modified and expanded to generate nourishment for the intrauterine progeny (Tobe and Langley, 1978). The primary nutrients within tsetse milk are lipids and proteins with amino acids and sugars as minor components (Cmelik et al., 1969; Denlinger and Ma, 1974). To date, four proteins have been identified as components of the milk, and are generated within the milk gland (Attardo et al., 2006; Guz et al., 2007; Yang et al., 2010). Up to 15 mg of lipids, consisting mostly of triacylglycerol (TAG) and phospholipids are transferred from mother to larva each gonotrophic cycle (Cmelik et al., 1969; Denlinger and Ma, 1974). However, little is known about this process. Lipids for milk production are not generated in the milk gland, rather they are produced and stored in the fat body or are acquired directly from blood feeding (Langley et al., 1981; Tobe and Langley, 1978). The lipids from both sources are moved to the milk gland for incorporation into the milk secretion (Langley et al., 1981; Tobe and Langley, 1978). Currently, the factors responsible for lipid transport from the sites of nutrient uptake (digestive tract) or storage (fat body) through the hemolymph and to the milk gland have not been examined.

Lipophorins (Lp) are critical in insects for lipid transport between tissues (Ryan and van der Horst, 2000; Soulages and Wells, 1994; Shapiro et al., 1988; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002). The Lp gene is expressed as a single transcript that is cleaved into two separate proteins after translation (Sundermeyer et al. 1996; Atella et al. 2006). Lipid loading and unloading occurs at tissues expressing the lipophorin receptor (LpR) (Canavoso et al., 2001; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002). Lipophorin transitions from the unloaded high density lipophorin (HDLp) to the lipid-loaded low density lipophorin (LDLp); (Ryan et al. 1986; Arrese et al., 2001; Arrese and Soulages, 2010; Canavoso et al., 2001; Van der Horst et al., 2002). The primary lipid transported by insect lipophorin is diacylglycerol (Chino and Kitazawa, 1981; Chino et al. 1981; Arrese et al., 2001; Arrese and Soulages, 2010; Van der Horst et al., 2002). In some cases, this protein can act as a shuttle for other hydrophobic moieties, such as hydrocarbons, cholesterol, phospholipids and fatty acids (Chino and Gilbert, 1971; Katase and Chino, 1984; Fan et al., 2002; Sevala et al., 1999). Upon arrival at target tissues, the protein-lipid complex binds to LpR and lipids are unloaded either with or without endocytosis (Parra-Peralbo and Culi, 2011; Rodenburg and Van der Horst, 2005; Ryan and van der Horst, 2000; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002; Van Hoof et al., 2005). After unloading, Lp is recycled for subsequent lipid mobilization (Rodenburg and Van der Horst, 2005; Ryan and van der Horst, 2000; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002; Van Hoof et al., 2005). Lipophorin systems have been thoroughly characterized in blood feeding insects including the yellow fever mosquito, *Aedes aegypti* (Van Heusden, 1997; Sun et al. 2000; Cheon et al. 2001; Cheon et al. 2006), the malaria mosquito, *Anopheles gambiae* (Atella et al. 2006; Marinotti et al. 2006) and in the kissing bug, *Rhodnius prolixus* (Machado et al. 1996; Grillo et al. 2003; Pontes et al. 2002; Pontes et al. 2008), but little is known about lipophorin in tsetse. Tsetse lipophorin (GmmLp) was previously isolated. It contains two subunits, apolipoprotein-I (250kDa; Apolipo-I) and apolipoprotein-II (80kDa; Apolipo-II) and has a density of 1.11g/ml (Ochanda et al., 1991). This lipid protein complex consists of 49% lipids and 51% protein (Ochanda et al., 1991).

The focus of this study is to understand the mechanism of lipid movement during pregnancy and lactation in tsetse. In particular these studies focus on the role of GmmLp as the lipid carrier molecule during the tsetse reproductive cycle. We characterize the molecular biology of lipophorin and its receptor (GmmLpR), and examine expression of *gmmlp* and *gmmlpr* during pregnancy. Localization of GmmLpR was conducted to identify potential target tissues at which lipid loading and unloading occurs. Adult female hemolymph, uterine fluid, larval gut contents and larval hemolymph were examined for the presence of GmmLp to determine if this lipoprotein can facilitate direct transfer of lipids from mother to intrauterine offspring. Lastly, the physiological roles of GmmLp and Gmm LpR during pregnancy were assessed utilizing single stranded RNA based (siRNAi) knockdown. The putative roles of GmmLp and GmmLpR in the oogenesis and larvagenesis processes are discussed.

2. Materials and Methods

2.1. Flies

Colonies of *Glossina morsitans morsitans* at Yale University (New Haven, CT, USA) originated from a small population of flies originally collected in Zimbabwe. Flies are maintained at 24°C and 50–60% RH. Flies receive bovine blood meals via an artificial feeding system every 48h (Moloo, 1971). Mated female flies were collected for qPCR and western blotting according to developmental markers established in previous studies (Attardo et al., 2006; Yang et al., 2010). In addition, to differentiate between maternal and larval gene expression, progeny were removed from pregnant females and both samples (pregnant females and the larva) were analyzed individually.

2.2. Phylogenetic analysis of *gmmlp* apolipoprotein II and I

BLASTX analysis of tsetse cDNA and genomic read libraries at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/g_morsitans) were utilized to identify tsetse *gmmlp* sequences. Predicted protein sequences were aligned using ClustalX (Mega 4, Thompson et al., 1997) and formatted with BioEdit (Hall, 1999). Pairwise phylogenetic tree construction and bootstrap analysis (10000 replicates) were performed using the MEGA3 sequence analysis suite (Kumar et al., 2004).

2.3. Analysis of *gmmlp* and *gmmlpr* expression

Levels of *gmmlp* and *gmmlpr* 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 primer sequences utilized for *gmmlp* were (F – 5'-TTTGGCTCCGAAATGATGTTCTTGA -3' and R – 5'-TGTC AATTC CGGACGAAATTTGTTTGT-3') and for *gmmlpr*: (F – 5'-GGCCGAACGGCATTACATTGGA -3' and R – 5'-TTGACGACGTTGCGAACCATCA-3'). All treatments were normalized according to tsetse Tubulin (*gmmtub*) expression levels using gene specific primers (F – 5'-CCATTCCCACGTCTTCACTT -3' and R – 5'-GACCATGACGTGGATCACAG -3') and carried out in triplicate.

For tissue specific expression analysis, cDNA was prepared from 2 µgs of total RNA recovered from dissected tissues using Trizol (Invitrogen, Carlsbad CA) and the Superscript 3 cDNA synthesis kit (Invitrogen) according to manufacturers' specifications. Tissues isolated were Malpighian tubules, salivary gland, reproductive tract, fat body, ovary, milk gland, midgut and larva. PCR was conducted with gene-specific primer sets: *gmmlp* (F-5'-GGAATATGGTAAAGTGGTGG-3', R-5'-TTAGGAGCAAATGCAGGA-3') and *gmmlpr* (F-5'-GTATTGGTCGGATTGGGG-3', R-5'-TGGCTGACGATACGGATG-3'). As a control *gmmtub* were used (F-5'-TCGTTGACCATGTCTTGGTGT-3' and R-5'-

TAGTTCTCTTCAACTTCAGCCTCTT-3'). The PCR amplification conditions were 95°C for 3 min, thirty cycles of 30s at 95°C, 52 or 56°C for 1 min, and 1min at 70°C in a Bio-Rad DNA Engine Peltier Thermocycler (Hercules, CA).

2.4. Lipid quantification

Adult female hemolymph lipid levels were measured with a modified vanillin reagent assay. Hemolymph (2µl) was collected utilizing a pulled glass capillary tube using reverse pressure from five female flies. Samples were checked by microscopic analysis to ensure hemolymph was not contaminated with fat body cells. A portion of the combined samples (1 µl) was dried at 50°C in 5 ml glass test tubes for 2 days. Lipids were dissolved in sulfuric acid at 90°C for 10 min and allowed to cool to room temperature. The samples were then combined with 4ml of vanillin reagent (Van Handel, 1985), and the absorbance was measured at 525 and 490nm. Concentration was determined according to standard lipid levels.

2.5. Western blot and immunohistochemical analyses

Protein was isolated from flash frozen female flies and tissue samples utilizing a Trizol-based protocol modified to dissolve the protein pellets in cracking buffer (8M urea, 3M thiourea, 1% dithiothreitol (DTT) and 4% CHAPS). Equal volumes of protein from three flies were combined for each time point, and analyzed by standard western blot protocol (Attardo et al., 2006). Apolipo-II antisera used in this study were generated against *Aedes aegypti* Apolipo-II and LpR antisera against *Drosophila melanogaster* LpR. Antisera against Tubulin (GmmTub) and Milk Gland Protein (GmmMGP1) were previously generated against recombinant tsetse GmmTub and GmmMGP1 (Attardo et al., 2006). Analysis of GmmLp, GmmLpR and GmmTub was performed utilizing the protein equivalent of 1/100th of a fly per well. Blots were blocked overnight in PBS, 3% BSA and 0.5% Tween 20 (blocking buffer). ApoLipo-II, DmLpR and GmmTub antisera were diluted 1:5,000 in blocking buffer. GmmMGP1 antisera was utilized at 1:20,000 (Attardo et al., 2006). Signals were visualized with Supersignal West Pico Substrate (Pierce, Woburn, MA) on a Image Station 2000R (Kodak, New Haven, CT).

Immunohistochemical analysis utilizing DmLpR antisera was performed by horseradish peroxidase based staining. Tissues from pregnant flies carrying larva were microscopically isolated, fixed and stained as described (Attardo et al., 2006; Guz et al., 2007). Staining was performed with the Novared Peroxidase staining kit (Vector, Burlingame, CA) according to manufacturers protocol.

2.6. Assessment of GmmLp movement from mother to developing larva

To assess GmmLp movement directly between the mother and larva during pregnancy, hemolymph from adult females, uterine fluid, larval gut contents and larval hemolymph were collected. Fluid within the uterus (uterine fluid contains milk received from the mother and other fluids in the uterus) was collected by inducing parturition and collecting the fluid expelled immediately prior to parturition. Hemolymph and gut contents of 3rd instar larvae were collected by removing progeny from the uterus of pregnant females. Hemolymph was obtained by inserting a pulled glass capillary tube directly under the cuticle of the larva (after removal from the uterus) or the pregnant female (before larval removal), and observed microscopically to ensure fat body cells did not contaminate the samples. Larval guts, which contain recently secreted and partially digested milk from the mother, were removed by dissection and washed in PBS with 0.1% Tween to remove GmmLp contamination from larval hemolymph and contents were collected directly using a pulled glass capillary needle. Western blot analysis was performed using AaApolipo-II and GmmMGP1 antisera as described. Protein loading was assessed using Coomassie blue gel staining as the milk is devoid of proteins used for internal control (Attardo et al., 2006).

2.7. RNA interference of *gmmlp* and *gmmlpr*

cDNA clones for *gmmlp* and *gmmlpr* and a plasmid containing *gfp* served for PCR amplification: *gmmlp* T7 forward 5'-TAATACGACTCACTATAGGGAGAGAATATGGTAAAGTGGTGG-3', *gmmlp* T7 reverse 5'-TAATACGACTCACTATAGGGAGATTAGGAGCAAATGCAGGA-3', *gmmlpr* T7 5'-TAATACGACTCACTATAGGGAGATGAAGGTTGGATGTATTGG-3', *gmmlpr* T7 5'-TAATACGACTCACTATAGGGAGA GTCCGGTGAATTTATTGCT-3', *gfp* T7 forward 5'-TAATACGACTCACTATAGGGTCAGTGGAGAGGGTGAAG-3', *gfp* T7 reverse 5'-TAATACGACTCACTATAGGCTAGTTGAACGGATCCATC-3'. Both primers contain the T7 promoter sequence at 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 the QIAquick PCR purification kit (Qiagen, Valencia, CA) and validated by sequencing at the DNA Analysis Facility at Yale University. Sense and antisense RNA were synthesized using the MEGAscript RNAi Kit (Ambion, Austin, TX), and subsequently purified using a RNeasy Mini Kit (Qiagen, Valencia, CA), and suspended in RNase-free water. The Block-iT Dicer RNAi kit (Invitrogen, Carlsbad, CA) was used to treat dsRNA to generate siRNA. Concentration was determined spectrophotometrically, and adjusted to 200–300 ng/μl. Each fly was injected with 1 μl siRNA 12h after adult emergence when assessing oocyte and embryonic development and then again at 10d when measuring abortion rates and overall gonotrophic cycle length. Expression levels normalized to *tubulin* were determined utilizing qPCR as described. Knockdown effects on larval and oocyte/embryonic development were characterized by dissecting flies and microscopically analyzing their reproductive status according to the previously established tsetse developmental classification (Attardo et al., 2006). During oocyte/embryonic development, the following values were assigned to each female, 0 = No oocyte/embryo/larva, 1 = Stage 1 oocyte, 2 = Stage 2 oocyte, 3 = Stage 3 oocyte, 4 = embryo and 5 = first instar larva. Each treatment represents three groups of five flies.

3. Results

3.1. Phylogenetic analysis of GmmLp

Partial sequences for *gmmlp* and *gmmlpr* were identified by previous EST projects (Attardo et al., 2006). Based on the translated sequence, an amino acid alignment with other insect Lp genes was conducted. The putative GmmLp contains two predicted peptides, Apolipoprotein II and Apolipoprotein I (Supplemental Figure 1). Phylogenetic analysis of the putative GmmLp shows that this sequence is most closely-related to those of *Drosophila sp.* and other Dipterans (Supplemental Fig. 2). Partial GmmLpR sequences were previously analyzed by Ciudad et al. (Ciudad et al., 2007), and also found to be most closely-related to other Dipterans.

3.2. Transcript levels of *gmmlpr* and *gmmlp* levels throughout pregnancy

A temporal qRT-PCR analysis was conducted to determine *gmmlpr* and *gmmlp* expression in whole females during the first two gonotrophic cycles. To determine developmental stages, fertile 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) according to Attardo et al. (Attardo et al., 2006) and illustrated in (Fig. 1A). Transcript levels of *gmmlpr* were relatively constant throughout the first gonotrophic cycle with the exception of a slight decline at the beginning of larvigenesis. During the second gonotrophic cycle *gmmlpr* levels

were 30–50% higher than average during the first gonotrophic cycle (Fig. 1B). Transcript levels of *gmmlp* were significantly higher at the end of oogenesis than they were in teneral newly eclosed flies. When measured from flies undergoing larvigenesis, *gmmlp* levels positively correlated with larval development followed by a drop in transcript levels after parturition (Fig. 1C). A more detailed analysis immediately after parturition showed that *gmmlp* levels declined by 50% after larviposition, and then increased again at 144h (Fig. 1D). This observation correlates with the beginning of larval development within the uterus. When *gmmlp* levels were measured from 1st, 2nd and 3rd instar larva and their corresponding mothers, a progressive increase was noted in each successive instar while levels in the mother were not significantly different (Fig. 2). Thus the increase in *gmmlp* levels noted in whole flies, which contain both the maternal and larval transcripts, results from the increase in larval transcript levels. This suggests that after the initial increase at the end of oogenesis, *gmmlp* levels remain relatively constant in the female throughout the gonotrophic cycle.

3.3. Tissue specific localization of *gmmlp* transcripts and protein

RT-PCR and western blot analysis of *gmmlp* and GmmLpR detected corresponding transcripts and protein within the midgut, head, fat body/milk gland, ovary/oocyte, and larva, respectively. No signal was detected in the salivary glands, Malpighian tubules or reproductive tract (Fig. 3A). Immunohistochemical analysis of the milk gland along with other components of the reproductive organs and fat body revealed that GmmLpR is associated with the milk gland cells and spermatheca (Fig 3B), and the fat body (Fig. 3C). The normally clear region surrounding the spermatheca tissues and the associated tubules test positive for LpR (Fig. 3B). No GmmLpR was detected in the reproductive tract or the larval cuticle present in the background of Fig. 3B.

3.4. Gmmlp tissue specificity and absence of GmmLp transfer from mother to offspring

RT-PCR analysis of *gmmlp* indicated the presence of corresponding transcripts in the fat body of adult females and larvae. The larval expression is likely occurring in larval fat body (Fig. 4A). To determine if GmmLp can function to directly transfer lipids from mother to her intrauterine offspring, western blot analyses of hemolymph from pregnant females and larva, uterine fluids and larval gut contents was performed. Lane loading was confirmed by total protein staining with Coomassie blue, where two bands are present for putative apolipoproteins corresponding to expected sizes of apolipoprotein I (250kDa) and apolipoprotein II (75KDa). The western analysis using an Apo-lipoprotein II antibody revealed that apolipoprotein II is only present in adult and larval hemolymph and absent from the uterine fluids and larval gut contents, which contain the milk secretions of the milk gland organ (Fig. 4B). Major milk gland protein (GmmMGPI) which is only found in the milk gland and milk secretions was included as a control and was detected only in the uterine fluids and larval gut contents (Fig. 4B). These results suggest that GmmLp is not transferred from the female hemolymph into the developing larva through the uterus. In addition, GmmLp is not incorporated into the milk as a lipid transporter, due to its absence in the uterine fluids and larval gut contents. Based on gene expression and protein localization studies, GmmLp appears to be expressed in the fat bodies of the adult female and her immature intrauterine progeny independently during development.

3.5. Functional analysis of GmmLp and GmmLpR by RNAi knockdown

To determine the functional roles of GmmLp and GmmLpR during development, we performed gene knockdown experiments. Analysis 5 days post treatment revealed that levels of *gmmlp* and *gmmlpr* were reduced, by about 75 and 60%, respectively, in the mother (Fig. 5A). No differences were noted for expression levels of either gene within the intrauterine larvae (data not shown). Hemolymph lipid levels after *gmmlp* knockdown were reduced by nearly 50% while no significant effect was observed after *gmmlpr* knockdown (Fig. 5B).

Oocyte and embryonic development were also affected following *gmmlp* knockdown (Fig. 5C). In the *gmmlp* siRNA treatment group (*siLp*) most females were carrying Stage 3 oocytes, while control flies (injected with *siGFP* or PBS-only) had completely ovulated and were gestating embryos (Fig. 5C and D). No significant difference was noted in progeny development after *gmmlpr* knockdown (*siLpR*) (Fig. 5C). Knockdown of *gmmlp* however resulted in about an 8 day extension of the first gonotrophic cycle (Fig. 5E). This extended duration results from approximately a 2d delay in oocyte development (Fig. 5C) and 6d delay in larval development (Fig. 5E). In addition, knockdown of *gmmlpr* resulted in about a 3 day delay of larval development (Fig. 5E). The abortion rate after *gmmlp* knockdown was nearly 20% (24.5% vs. 5.1%; Fig. 5F) higher than control individuals (*siGFP* and PBS-injected) or after knockdown of *gmmlpr*. Thus, it appears that interference with the lipophorin system causes a significant negative effect upon progeny development, where *Lp* knockdown seems to delay both early oocyte and larval development and *LpR* knockdown impacts only larval development.

4. Discussion

Here we show that lipophorin is synthesized in the *G. m. morsitans* fat body tissue and acts as the primary shuttle for lipids in the adult hemolymph during pregnancy. In contrast the receptor *GmmLpR* has a wider expression profile, present in the head, fat body, midgut, milk gland, ovaries and spermatheca. These are all common sites for lipid transfer based on findings in other insect species (Arrese et al., 2001; Canavoso et al., 2001; Ryan and van der Horst, 2000; Soulages and Wells, 1994; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002; Van Hoof et al., 2005). Lipophorin is not transferred to the developing intrauterine larva in mother's milk secretions. Instead the larva synthesizes its lipophorin independently to transport lipids from maternal milk secretions in the larval gut. Thus, lipids in female milk secretions apparently require a novel transport mechanism independent of *GmmLp*.

Our results show that knockdown of *gmmlp* expression had a significant impact on hemolymph lipid levels, oocyte development, larval deposition and abortion rate. However, knockdown of *GmmLpR* did not alter hemolymph lipid levels and oocyte development in a significant way, but did delay larval development. It is possible that lipophorin may be capable of transferring lipids directly through the cell membrane without the help of the receptor. Another possibility is that the level of the knockdown achieved for *gmmlpr* may be insufficient to disrupt lipid transfer. In the case of oocytes, this could prevent the observation of a significant oocyte phenotype. Finally, it is also possible that the receptor protein may be very stable and proteins synthesized prior to the siRNA treatment may still be at a high enough concentration to provide the lipids necessary for oocyte development in knockdown flies (Dantuma et al., 1998; Parra-Peralbo and Culi, 2011; Ryan and van der Horst, 2000; Van der Horst and Rodenburg, 2010; Van der Horst et al., 2002). The phenotype during larvigenesis after *gmmlpr* knockdown likely occurs as the amount of lipids transferred during larval development is much higher than the amount transferred during oogenesis.

Recent work shows that lipophorin can cross the blood brain barrier (BBB) in *Drosophila* (BBB) (Brankatschk and Eaton, 2010). The ability of the protein to traverse the BBB suggested the possibility that this lipid shuttle has the potential to move fat directly from the mother to the intrauterine offspring. We reasoned that *GmmLp* could be taken up by the milk gland from the hemolymph and transferred to the larva in the milk or alternatively *GmmLp* could pass directly through the uterine wall to be taken up by the larvae (Langley et al., 1981; Tobe and Langley, 1978). Detection of *GmmLp* in the maternal and larval hemolymph only and its absence from uterine fluids or larval gut contents, indicates

however that GmmLp is not transferred from the mother to developing larva via the milk gland or through uterus. This indicates that GmmLp pools are synthesized separately and remain distinct throughout pregnancy between mother and progeny. As such, it appears that the milk gland secretions are the sole route for nutrient provisioning to the larva. Once lipids are transferred into the milk gland cells by GmmLp it is likely that another biochemical molecule promotes their transfer in the milk to feed the developing larva (Cmelik et al., 1969).

Based on the localization of GmmLp and GmmLpR transcripts and proteins, we have developed a tentative model for lipid mobilization in tsetse during pregnancy with an emphasis on the role of GmmLp for transport and GmmLpR as an indicator of tissues involved in lipid transfer (Fig. 6). Biochemical studies will be necessary to thoroughly assess lipid classes transported by GmmLp to the different tissues, the milk gland in particular. As an obligate hematophagous insect, tsetse flies acquire lipids and all other nutrients via blood feeding. Dietary lipids are absorbed from digested blood and loaded to lipophorin at the midgut/hemolymph interface. Loaded lipophorin complexes travel through the hemolymph and unload lipids at the fat body for storage, ovaries for oogenesis and the milk gland for milk production. The function of GmmLpR in the spermatheca is not characterized but suggests that lipids may also be important for sperm storage, longevity or mobilization. Stored lipids from the fat body are mobilized to the milk gland, and are critical for larval nutrition during periods of starvation. Once lipids are unloaded at the milk gland cells, they are transferred to the larval progeny not by lipophorin but by another carrier protein. We hypothesize that the abundant milk gland protein (GmmMGPI) could be a candidate molecule for this function within the milk and studies are ongoing to test this hypothesis (Attardo et al., 2006; Yang et al., 2010).

Previous studies have addressed the role of lipophorins during oocyte development in the malaria mosquito (*Anopheles gambiae*), the yellow fever mosquito (*Aedes aegypti*), and the German cockroach (*Blattella germanica*). In *A. gambiae*, AgLp is taken into the oocyte by receptor-mediated endocytosis resulting in the formation of fat vesicles throughout the oocyte (Atella and Shahabuddin, 2002; Atella et al., 2006). Within the yellow fever mosquito, AaLp is also utilized as a yolk protein precursor (Sun et al., 2000). In *B. germanica*, RNAi of *bglpr* knocked down transcript and proteins levels, but had no apparent phenotype (Ciudad et al., 2007). A similar result was noted here, as no difference in oocyte development was observed after GmmLpR knockdown, but we did note an effect during larval development. While GmmLpR knockdown failed to reduce oocyte development, GmmLp knockdown led to a significant delay in oocyte development. Delayed oocyte development after GmmLp reduction is likely due to either reduced storage lipids and hydrocarbons available for egg development (Fan et al., 2002; Van der Horst et al., 2002) or reduced incorporation of GmmLp into yolk granules (Sun et al., 2000). Currently, no studies have addressed the role of tsetse lipophorin during intrauterine larval development. Here, we show that *gmmlp* knockdown results in lower hemolymph lipid levels leading to delayed larval development and increased larval abortion, indicating that GmmLp is critical to all aspects of tsetse progeny development.

Reproduction in tsetse has similar physiological characteristics to mammalian reproduction in terms of intrauterine development and subsequent lactation after birth. The uterus provides a selective barrier in both mammalian and tsetse systems with the milk gland functioning as the morphological equivalent to the placenta allowing the mother to provide nutrients to the developing offspring within the uterus. However, the products and mechanisms of the milk gland function in tsetse is more orthologous to mammary gland function in respect to nursing newborns (Ma et al., 1975; McManaman and Neville, 2003). In particular, the lactation secretions, in terms of lipid content and protein function, are

similar (Cmelik et al., 1969; Ferris and Jensen, 1984; Hamosh et al., 1985; Koletzko et al., 2001; Lammi-Keefe and Jensen, 1984; McManaman and Neville, 2003; Neville and Picciano, 1997; O'Donnell et al., 2004). Additionally, the milk represents a source of symbiont transfer from the mother to her progeny (Denlinger and Ma, 1975; Lara-Villoslada et al., 2007; Martin et al., 2003)

Due to similarities in nutrient provisioning, such as the functional orthology of milk proteins between species and lipid transfer to milk producing tissues, aspects of tsetse lactation are comparable to mammalian lactation. In both cases, lipids are only transferred from a single organ (mammary and milk gland) to feed the progeny. The lipid transfer mechanisms from nutrient stores (i.e. fat tissue) through the hemolymph/blood to milk-producing tissues between tsetse and mammalian systems are similar except for a few differences. Mammals mobilize lipid as free fatty acids bound to albumin and lipoprotein complexes (low density lipoproteins, LDLs). The majority of lipid transfer in insects is restricted to a single lipoprotein (lipophorin) (Barber et al., 1997; McManaman and Neville, 2003; Ryan and van der Horst, 2000; Van der Horst and Rodenburg, 2010). In addition, mammalian LDLs are not recycled as are those of insects. A mammalian lipoprotein lipase breaks down the entire LDL complex to release the lipoprotein-associated lipids in target tissues (Barber et al., 1997). In insects, the lipids are hydrolyzed from the protein and then lipophorin is returned to the hemolymph (Ryan and van der Horst, 2000; Van der Horst and Rodenburg, 2010). Another difference in lipid mobilization between insects and mammals is the lipid products mobilized by lipoproteins. Mammals transport triacylglycerol while insects transport diacylglycerol (Barber et al., 1997; McManaman and Neville, 2003; Ryan and van der Horst, 2000; Van der Horst and Rodenburg, 2010). Lipids acquired from the blood/hemolymph by the mammary/milk glands are processed, packaged and secreted primarily as triglycerides (> 95%) in addition to a variety of other hydrophobic components consisting of phospholipids and cholesterol (Barber et al., 1997; Cmelik et al., 1969; Hamosh et al., 1985; McManaman and Neville, 2003; Ryan and van der Horst, 2000; Van der Horst and Rodenburg, 2010). Tsetse pregnancy represents the compression of intrauterine development and lactation in mammalian systems into a single physiological stage. However, functional orthology of the systems has resulted in development of comparable mechanisms between the reproductive biology of tsetse flies and mammals. Comparative analysis of reproductive biology of these two systems could provide insight into the critical functions lactation serves during early organismal development. Insights derived from comparisons between lactation systems could be used in future analyses of lactation in general, tsetse reproductive biology and in the development of novel methods of tsetse control.

Supplementary Material

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Fig. 1.

Time course of *gmmlp* and *gmmlpr* expression during the first two tsetse gonotrophic cycles. A. Illustration of the tsetse reproductive tract and microscopic visualization of the developmental stages represented in the time course. B. and C. represent *gmmlp* and *gmmlpr* transcript levels during the different developmental stages in the first gonotrophic cycle and beginning of second gonotrophic cycle as illustrated in (A). D. represents *gmmlp* levels measured at 24 hour intervals post parturition. Levels of *gmmlp* and *gmmlpr* 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). All qPCR assays

were carried out in triplicate. Significant differences were determined by ANOVA followed by Tukey's test.

Fig. 2. *gmmlp* expression in tsetse fly female and her developing larva throughout pregnancy determined by qPCR expression. Triangle, *gmmlp* expression in pregnant female with larva; square, *gmmlp* expression in pregnant female without larva; diamond, *gmmlp* expression in larva removed from pregnant female. E, embryo; L1, 1st instar larva; L2; 2nd instar larva; L3, 3rd instar larva; P, immediately after parturition.

Fig. 3. Spatial analysis of GmmLpR. A. RT-PCR and western blot localization of LpR protein and transcript in the Malpighian tubules (MT), salivary gland (SG), reproductive tract (RT), head (H), Ovary (O), fat body/milk gland (FM), midgut (M) and larva (L). B. Immunohistochemical localization of GmmLpR after removal of fat body (left panel, DmLpR antisera as primary antibody, right panel, no primary antibody). C. Immunohistochemical analysis of the fat body (left panel, GmmLpR antisera as primary antibody, right panel, no primary antibody).

A.

B.

Fig. 4.

RT-PCR analysis of *gmmmlp* tissue specificity and western blot analysis of GmmLp localization in maternal hemolymph, uterine fluid, larval hemolymph and gut contents. A. RT-PCR localization of *gmmmlp* in the Malphigian tubules (MT), salivary gland (SG), reproductive tract (RT), fat body (FB), Ovary (O), milk gland (MG), midgut (M) and larva (L). Each localization was conducted on three biological replicates. B. Maternal hemolymph, uterine fluid, larval hemolymph and larval gut contents were collected from pregnant females carrying 3rd instar larva. Total protein levels per lane were shown by Coomassie blue staining of the membrane. L, larva; ♀, female.

Fig. 5. Phenotypic effects of *gmmlp* and *gmmlpr* suppression. A. RNA interference of *gmmlp* and *gmmlpr* 5d after siRNA injection and adult emergence, respectively. Light gray, *gmmlp*; dark gray, *gmmlpr*. Each treatment is the mean $\pm$ SE of five flies. B: Hemolymph lipid content. Each treatment represents mean $\pm$ SE for three groups of five flies. C: Oocyte and embryonic development. D: Examples of development after *gmmlp* knockdown compared to *siGFP*-injected individuals. E. Larviposition: Each treatment represents mean $\pm$ SE for three groups of 15 flies. F. Abortion: Each treatment represents mean $\pm$ SE for three groups of 15 flies. Transcript data represents the mean normalized *gmmlp* and *gmmlpr* transcript level

from 5 flies. Values were compared using ANOVA followed by Tukey's test ($P < 0.05$). *, indicates statistical difference compared to controls.

Fig. 6.

Synopsis of lipophorin-based lipid mobilization during tsetse fly pregnancy. All nutrients are acquired from blood since it is the only nutrient source for tsetse flies. Lipophorin (GmmLp) allows movement of lipids through the hemolymph between sites of lipophorin receptor (GmmLpR). Thus, GmmLp allows movement of lipids from tissues generating lipids (midgut and fat body) to the ovary for oocyte generation and the milk gland to feed the developing larva. The spermatheca and ovary represent two other location of GmmLpR expression, which are likely critical for tsetse reproduction. GmmLp is not incorporated directly into the milk and cannot pass through the uterus, indicating that most, if not all, lipids need to come directly through the milk gland.