Published in final edited form as:
J Insect Physiol. 2007 July ; 53(7): 715–723.

Molecular aspects of transferrin expression in the tsetse fly (*Glossina morsitans morsitans*)

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Abstract

Iron is an essential element for metabolic processes intrinsic to life, and yet the properties that make iron a necessity also make it potentially deleterious. To avoid harm, iron homeostasis is achieved via proteins involved in transport and storage of iron, one of which is transferrin. We describe the temporal and spatial aspects of transferrin (GmmTsf) expression and its transcriptional regulation in tsetse where both the male and female are strictly hematophagous. Using Northern, Western and immunohistochemical analysis, we show that GmmTsf is abundant in the hemolymph and is expressed in the adult developmental stages of male and female insects. It is preferentially expressed in the female milk gland tubules and its expression appears to be cyclical and possibly regulated in synchrony with the oogenic and/or larvigenic cycle. Although no mRNA is detected, GmmTsf protein is present in the immature stages of development, apparently being transported into the intrauterine larva from the mother via the milk gland ducts. Transferrin is also detected in the vitellogenic ovary and the adult male testes, further supporting its classification as a vitellogenic protein. Similar to reports in other insects, transferrin mRNA levels increase upon bacterial challenge in tsetse suggesting that transferrin may play an additional role in immunity. Although transferrin expression is induced following bacterial challenge, it is significantly reduced in tsetse carrying midgut trypanosome infections. Analysis of tsetse that have cured the parasite challenge shows normal levels of *GmmTsf*. This observation suggests that the parasite in competing for the availability of limited dietary iron may manipulate host gene expression.

Keywords

Viviparous reproduction; larvigenesis; oogenesis; tsetse; transferrin; trypanosome

1. Introduction

Human African trypanosomiasis (HAT) kills thousands of people each year in sub-Saharan Africa (Barrett 1999;Ekwanzala et al. 1996). The disease is caused by the African trypanosome, a protozoan parasite, and is transmitted to the mammalian host by the tsetse. Both adult male and female tsetse are strict blood feeders and are capable of parasite transmission. Tsetse is unusual among insects in having a viviparous reproductive biology, where the entire larval development (1st to 3rd instar) is intrauterine and a fully mature larva is deposited and pupates immediately. A key component of tsetse's viviparous biology is the accessory gland (milk gland), an organ consisting of tubules that intertwine throughout the fat body in the abdominal

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cavity and coalesce at the opening of the uterus where the developing larva feeds. During its intrauterine life, the larva receives nutrients in the form of “milk” via the secretions from mother’s accessory glands.

We have recently shown that an important component of the “milk” is a protein called the milk gland protein (GmmMGP), which belongs to the lipocalin family (Attardo et al. 2006b). Expression of GmmMGP in the mother may be subject to both transcriptional and translational control according to the larvigenesis process, with peak levels detected during the 3rd instar development. The abundant GmmMGP protein may either bind small hydrophobic molecules, a typical function of the lipocalin family, and/or may itself serve as the source of amino acids for the developing larva. The protein is synthesized in the adult female and is transported to the intrauterine larva where it can be detected during the larval and pupal stages. Tsetse relies solely on a diet of vertebrate blood for the entirety of its development. To enable survival on a single diet, they have established mutualistic relationships with symbiotic bacteria in order to supplement their diet with essential nutrients necessary for fecundity (Aksoy 2000).

In addition to serving as the source of energy for vital processes, the host blood meal provides iron for all developmental stages of tsetse, adult, larvae and pupae, as well as the inhabitant microbial flora and in some cases with the parasite trypanosomes in infected flies. While iron is an essential element for the metabolic processes important to tsetse and its inhabitants, it can also be potentially deleterious and needs to be closely regulated especially in hematophagous insects (Dunkov and Georgieva 2006;Nichol et al. 2002). Sensitive regulation of iron metabolism in the tsetse not only affects host reproductive success, but may also potentially influence symbiotic fitness and parasite survival in the fly. Previously, we had described the molecular characteristics of tsetse’s protein homologues involved in iron-binding, transport and storage from a fat body/milk gland tissue specific gene discovery project (Attardo et al. 2006a;Strickler-Dinglasan et al. 2006). Among these are the iron-binding glycoproteins, transferrin and ferritin, and a putative transmembrane transporter with strong similarity to *Drosophila melanogaster* NRAMP2 (DMT1). The molecular characterization of the 2108 bp full-length *GmmTsf* cDNA predicted a secretory protein with a molecular mass of about 72 kDa (Strickler-Dinglasan et al. 2006). The putative GmmTsf has apparently retained the signature amino acids found conserved in invertebrate transferrins and similarly lacks iron-binding residues in its C-terminal lobe when compared to vertebrate transferrins (Dunkov and Georgieva 2006;Harizanova et al. 2005;Jamroz et al. 1993;Yoshiga et al. 1999). Despite the strict blood feeding requirement seen in both sexes in tsetse adults, the expression of *GmmTsf* was found to be female specific and was restricted to fat body/milk gland tissue fraction and absent from the midgut.

Here we report on a detailed analysis of the temporal expression of *GmmTsf* mRNA and protein, as well as the tissue and sex-specific nature of its synthesis during development. Using a transferrin specific antibody generated against recombinant GmmTsf, we compare its protein levels during development in different tissues in male and female flies and further localize its synthesis via immunohistochemical analysis. We also report on the immune-related expression profile of *GmmTsf* from pathogen challenged flies and from flies with midgut trypanosome infections. We discuss the implications of our findings with respect to transferrin’s postulated role as an iron-binding, vitellogenic and immune-responsive protein.

2. Materials and methods

2.1. Biological material

The *Glossina morsitans morsitans* (*G. m, morsitans*) colony maintained in the insectary at Yale University was originally established from puparia from fly populations in Zimbabwe. Newly emerged flies are separated by sex and mated at three to four days post-eclosion. Flies are

maintained at 24±1°C with 50-55% relative humidity, and receive defibrinated bovine blood every 48h using an artificial membrane system (Moloo 1971). For expression analysis upon immune challenge, newly emerged teneral flies were exposed to a blood meal supplemented with *in vitro* cultured 10^5 *Trypanosoma brucei rhodesiense* Ytat1.1 procyclic form parasites/ml. Newly emerged teneral flies also were given a blood meal containing 10^5 *E. coli* K12 cells.

2.2. Northern blot analysis

Newly eclosed females were mated at day 5 and collected in groups of three per day for the 30 day time course and snap frozen in liquid nitrogen. Total RNA was isolated from individual flies using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Ten micrograms of RNA from each sample was analyzed on a 1.5% agarose/formaldehyde gel and transferred to a nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ) by capillary blotting. Probes were generated by PCR Dig Probe Synthesis Kit (Roche Applied Science, Indianapolis, IN) utilizing gene specific primers *GmmTsf*: TrfR 5'-AGCATCTCGAGGTCTTTCTCTTGGACACATTCCA-3' and TrfF5'-ATCATGAGCTCTCAGAGGCCGGTATTAATATGC-3' and for *GmmTub*: TubR 5'-GACCATGACGTGGATCACAG-3' TubF 5'-CCATTCCCACGTCTTCACTT-3' using the following program 95 °C for 3 mins, 95 °C for 30 sec, 55 °C for 45 sec, 72 °C for 2 mins for 35 cycles and 72 °C for 7 mins. Blots were prehybridized at 42 °C for at least four hours and were hybridized with DNA probes overnight at 42 °C. Low stringency washes were performed with 2X SSC with 0.1% SDS for 2 times for 5 minutes at 42°C under constant agitation. High stringency washes were performed with 0.5 X SSC with 0.1% SDS at 68°C for 2 times for 15 minutes under constant agitation. Chemiluminescent detection of blots was performed with Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science) according to manufacturer's instructions. All blots were stripped and rehybridized with *GmmTub* probe as an internal loading control and hybridization signals for *GmmTsf* were normalized to the *GmmTub* signal using Kodak 1D 3. 6. 1. Imaging Software.

To analyze *GmmTsf* expression in mothers and their intrauterine larvae during the course of pregnancy, offspring were dissected from the uterus of the pregnant females during different stages of pregnancy; i.e., 1st instar larvae, 2nd instar larvae and 3rd instar larvae, and RNA was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, CA).

Tissue specific expression analysis was accomplished using midgut, fatbody/milk gland, reproductive tract and carcass dissected from mated flies during all stages of the reproductive cycle. Microscopically dissected tissues were collected in phosphate buffered saline (pH 7.4) and RNA was isolated using the TRIzol protocol.

To detect impact of trypanosome infection on *GmmTsf* transcript abundance, trypanosome infections were established in adult flies by challenging newly emerged (teneral) flies with a blood meal containing 1×10^6 Ytat 1.1 cells/ml supplemented with 0.05 M N-acetyl glucosamine. At 22-24 days post infection and 48 hr post routine blood feeding, midguts were dissected and microscopically examined for parasite infections and RNA was prepared from normal, trypanosome infected and self-cured females using the TRIzol® reagent.

2.3. Antibody creation

Anti-GmmTsf polyclonal antibodies were generated against a bacterially-expressed 6x His-tagged recombinant protein. Primers were designed to amplify the mature coding region from *GmmTsf* cDNA clone and included restriction sites to facilitate directional, in frame cloning into the pET-28a 6xHis-tag expression vector (Novagen, Madison, WI). The Reverse Primer with the XhoI site was 5'-AGCATCTCGAGGTCTTTCTCTTGGACACATTCCA and the Forward Primer with the SacI site was 5'-

ATCAT**GAGCTC**TCAGAGGCCGGTATTAATATG. The pET-28a–*GmmTsf* construct was transformed into the BL21 *E. coli* strain, and recombinant protein expression was induced by treatment of cultures with 100 μM IPTG. Bacteria were lysed by sonication and products were analyzed by SDS-PAGE. Recombinant protein was found predominantly in the insoluble fraction as inclusion bodies. Inclusion bodies were made soluble in binding buffer in the presence of 6M urea and purified by batch protocol using nickel resin under denaturing conditions protocol (Novagen His-Bind Kit). Recombinant proteins were subsequently purified by SDS-PAGE gel, and gel slices were provided for commercial antisera production (Cocalico Biologicals). Antisera were tested by Western blot analysis using recombinant proteins and female tsetse homogenates.

2.4. Western blot analysis

Mated flies for the 10-day protein expression time course were collected in groups of three per day and snap frozen individually. Early and late pupae were collected and snap frozen for protein extraction. Total protein extraction was performed according to previously published protocols (Park et al. 2006). Equal volumes of protein were combined from each of the three flies to generate a single sample for each day. Western blot analysis of the samples was performed following standard protocols (Sambrook and Russell 2001). The equivalent of $1/400^{th}$ of a fly was loaded in each well. Blots were prehybridized for at least four hours to overnight in 1X PBS, 3% BSA and 0.05% Tween 20. GmmTsf specific antisera were used at a concentration of 1:20000 in blocking buffer (3% BSA, 0.05% Tween 20 and 1X PBS). The blots were also hybridized to *Drosophila* Actin specific antibodies at a dilution of 1:1000 as a loading control (Abcam, Cambridge, MA). Signals were visualized using the supersignal west pico substrate (Pierce, Woburn, MA) on a Image Station 2000R (Kodak, New Haven, CT).

To confirm tissue specificity of GmmTsf, tissues were dissected in 1xPBS (pH 7.4) from females and males including carcass, fatbody and milk gland, digestive tract, reproductive tract and larvae separately to perform a western blot. Fly tissues were homogenized in equal volumes of cracking buffer (4M Urea, 2% SDS, 125 mM Tris-HCl pH 6.8 and 5% Beta-mercaptoethanol). The equivalent of 1/400th of the total protein from each sample was loaded per well.

The sub-tissue specificity analysis was performed on the reproductive tissues of females and males. The female reproductive tract was dissected into its smaller component parts: uterus, spermathica, active ovary, and inactive ovary. The testes were removed from males. Hemolymph samples were obtained by cutting off the wing of anesthetized male and female flies and collecting hemolymph from the site of the wound with a 1-20 ul pipette. Hemolymph was added directly to cracking buffer. Tissue samples from five individuals were combined and homogenized in equal volumes of cracking buffer. The equivalent of one flies worth of tissue was loaded per lane.

To detect GmmTsf in immature developmental stages, individual pregnant females were dissected in 1xPBS (pH 7.4) to remove the developing embryo or larvae from the uterus. The carcass and immature offspring were snap frozen and homogenized in cracking buffer. The equivalent of $1/400^{th}$ of a fly, embryo or larvae was loaded per well. Western blotting was performed as described above.

2.5. Immunohistochemistry

Immunohistochemical analysis of GmmTsf was performed using the antibodies generated against Rec-GmmTsf. Fat body, milk gland and reproductive tract were isolated from pregnant females. Tissues were fixed in 4% paraformaldehyde/PBS for 24 hours. Tissues were subjected to serial ethanol washes (0%, 20%, 40%, 60%, 80%, 100%, 80%, 60%, 40%, 20%, 0% for at

least 15 mins per wash) and then bleached with 30% hydrogen peroxide for two hours to remove endogenous peroxidase activity. They were then treated with blocking solution, primary GmmTsf (1:20000) antibodies, and horseradish peroxidase conjugated secondary antibodies (1:20000) with appropriate washes between treatments (Sillitoe and Hawkes 2002). Tissues were stained with the Novared Peroxidase staining kit (Vector, Burlingame, CA) according to manufactures protocols for antigen staining, and then analyzed microscopically.

2.6. qRT-PCR analysis of GmmTsf expression

Total RNA was prepared from five *E. coli* (10^6 cells/ml) and Ytat1.1 (10^6 cells/ml) challenged flies 24 h post infective blood meal acquisition. Similarly, RNA was prepared from five Ytat1.1 infected fertile females 22-24 days post acquisition of parasite infected meal and compared to age-matched female controls. For each analysis, a minimum of five normal individuals was included in the qRT-PCR analysis. RNA isolation was performed using the Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Samples were treated with RNase-free Turbo DNase I (Ambion) for 30 min. and 1μg of total RNA was used for cDNA synthesis using the Superscript II reverse transcriptase kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA). For qRT-PCR standard construction, inserts were cloned into the pGEM-T easy vector system (Promega, Madison, Wis.) using standard cloning techniques (Sambrook and Russell 2001). Transcript quantification was performed on the I Cycler Real Time detection system (Biorad) and data analyzed using software version 3.1. qRT-PCR was performed using the primer pair (GmmTsfF: AGTGACGGCTTTCTTTTCCA and GmmTsfR: ACCAAGCTGCCGCTTACTTA for 40 cycles using 58C as annealing temperature. Each sample was analyzed in triplicate and normalized to the internal control, tubulin mRNA. Transcript quantification for tubulin was performed using the primer pair GmmTubF: CCATTCCCACGTCTTCACTT and GmmTubR: GACCATGACGTGGATCACAG for 40 cycles using 58C as annealing temperature.

3. Results

3.1. Tissue and developmental stage specific expression of GmmTsf

We evaluated the transcript abundance of *GmmTsf* from different adult tissues including the midgut, fatbody/milk gland, reproductive tract and remaining carcass (Fig. 1A). In tsetse the milk gland tubules intercalate amongst the fat body tissue which is present throughout the abdomen, preventing clean isolation via microscopic dissection. Northern blot analysis showed that the majority of *GmmTsf* transcripts are expressed in the fatbody/milk gland fraction. Minimal amounts are detectable in the reproductive tract, larva and remaining carcass. Transcripts for *GmmTsf* could not be detected in the midgut.

In contrast to its restricted expression profile, a greater tissue tropism was observed for GmmTsf by Western analysis (Fig. 1B). GmmTsf protein was observed in the adult female fat body/milk gland fraction, the reproductive tissues and remaining carcass. GmmTsf could not be detected in the female digestive tract. In adult males GmmTsf was detected in the fat body and reproductive tract with the majority of the signal appearing in the remaining carcass. The wide tissue distribution of GmmTsf might result from its secretory nature as evidenced by the 5'- associated signal peptide sequence detected in the full-length cDNA as had been reported earlier (Strickler-Dinglasan et al. 2006). GmmTsf protein was also detected in larval samples. Four bands were observed at 72, 55, 47 and 42 kD in size. These bands may reflect proteolytic cleavage products generated within the larva after uptake from the mother.

As transferrin has been shown to be a vitellogenic protein in other insects, a closer examination of the hemolymph and reproductive tissues of male and female flies was conducted (Fig. 1C).

GmmTsf was detected in both male and female hemolymph as well as in the male testes and the female spermathica where the sperm is stored following insemination.

Oogenesis in tsetse begins before eclosion and only a single oocyte is developed at a time, thus we were interested in determining the presence of transferrin in the active and inactive ovaries. In protein samples from vitellogenically static and active ovaries, GmmTsf protein could only be detected in the active ovary (Lane 4 versus 5, respectively).

3.2 Temporal expression of GmmTsf

To determine the expression pattern of *GmmTsf* in relation to the processes of oogenesis and larvigenesis in pregnant females, a time course was performed over the first thirty days of adult life in tsetse. This analysis covered the first gonotrophic cycle and the beginning of the second. Transcript abundance was monitored by Northern blot analysis from equal amounts of RNA pooled from three whole flies per day (Fig. 2A). Transcript levels were quantitated and normalized against *GmmTub* to graphically represent transcript levels. Transferrin transcript levels increase steadily until the second oocyte has completed development and the first offspring is entering larval development in the uterus (Days 1-10). At this point a decline in transcript abundance was observed, followed by a steady increase in abundance through the birth of the first offspring, ovulation of the second oocyte and development of the third oocyte (days 11-21). The level of *GmmTsf* mRNA undergoes approximately 5-7 fold increase between the 1st and 3rd instar larval developmental period (days 13-21). One data set is presented here although this experiment was repeated at multiple times. Results indicated that *GmmTsf* transcription is regulated by female reproductive status.

Protein abundance was monitored in early and late pupae and into the first ten days post eclosion by western blot analysis of a fraction of total protein from three whole flies (Fig 2B). GmmTsf is abundant in early and late pupae and shows a banding pattern similar to that observed in larval GmmTsf (Fig. 1C), despite the absence of any detectable transcripts in this immature developmental period as shown previously in (Strickler-Dinglasan *et al.*, 2006). The protein detected in the pupae and in young adults immediately post eclosion may represent maternal GmmTsf obtained during larvigenesis as will be discussed in the next section. GmmTsf is found consistently in adult flies from newly eclosed flies to ten day old flies. In flies at nine and ten days old a second smaller GmmTsf band appears suggesting proteolytic processing. Nine to ten day old flies would be gestating the embryos of the first offspring of their reproductive cycle. It is possible that the cleavage is associated with embryonic development as we have observed in larval preparations before (Fig. 1B).

3.3. Transfer of GmmTsf from mother to larvae

To determine if transferrin is a component of the milk transferred to the developing larvae by the mother, we analyzed *GmmTsf* mRNA and protein levels in mothers and their intrauterine larvae during the course of pregnancy by northern and western blot analysis. Offspring were dissected from the uterus of pregnant females during the four stages of pregnancy (embryo, 1st instar larvae, 2nd instar larvae and 3rd instar larvae).

Transcripts for *GmmTsf* were only detectable in the mothers at all stages of larval development (Fig. 3A).

Western blot analysis shows that GmmTsf protein was undetectable in the embryo and weakly detectable in the mother during embryonic development when equal proportions of protein homogenates were analyzed (Fig. 3B). GmmTsf becomes detectible in the larvae during development of the 1st instar larvae and more prominent in the offspring during 2nd and 3rd instar development. This data provides strong support for the hypothesis that the intrauterine

larva obtains the transferrin protein from the mother via milk secretions. In the mother GmmTsf antisera recognizes two major bands at 72 kD and 47 kD, while in the larva the antisera recognizes three predominant bands at 72 kD, 55 kD, and 47 kD and a weaker fourth band at 42 kD. Two of the larval bands are the same size as those found in the mother. The two bands unique to the larva may reflect proteolytic cleavage products, a phenomenon that has been observed in transferrin in other insects.

3.5. Immunohistochemical localization of GmmTsf in pregnant females

We used immunohistochemical analysis to localize GmmTsf protein in the abdominal tissues of tsetse. As a control, fly tissues were stained with preimmune serum and secondary antibody (Fig. 4A,C). Immunohistochemical staining of flies pregnant with 2nd and 3rd instar larvae reveal specific staining of the milk gland tubules (Fig. 4B,D). Positive GmmTsf staining occurs throughout the milk gland tubules and in intense patches primarily at the tips of tubules traversing the fat body in the abdominal cavity. Staining is notably absent in the fat body tissue.

3.6. Expression of GmmTsf in immune challenged and parasite infected flies

The immune responsive expression profile of *GmmTsf* was determined by qRT-PCR analysis in flies following *per os* immune challenge by *E. coli* and trypanosome (Fig. 5A). Only the *E. coli* challenged group of flies indicated a statistically significant difference from the control group with approximately a 2-fold increase. The levels of *GmmTsf* expression were also evaluated from microscopically confirmed parasite infected flies analyzed three weeks post challenge and compared to the normal age-matched flies. Both qRT-PCR analysis (Fig. 5B) and Northern analysis (Fig. 5C) indicated a reduction of transferrin levels in flies with midgut infections, about 11 and 8, fold respectively. Furthermore, flies that had cured their parasite infections regained higher levels of transferrin mRNA (Fig. 5C, lane 2 versus 3, respectively), implying possibly that the trypanosomes may modulate host gene expression.

4. Discussion

Our results on the characterization of transferrin in tsetse suggest that it plays multiple physiological roles in immunity, iron metabolism and reproduction similar to that observed in other insects and vertebrates. However, the extreme physiological adaptations of the tsetse fly required for its blood specific diet and its viviparous reproductive strategy reveal further insights to the function of transferrin in insects in general as well as raise new questions.

GmmTsf expression in tsetse has similarities and differences with other insects in which it has been characterized. Northern analysis of tissue specificity shows that transferrin transcript is abundant in the fat body/milk gland fraction with lower levels of expression in the reproductive tract. Immunohistochemical experimental results indicate that this protein appears to be predominantly synthesized in the milk gland tissue of tsetse and not the fat body. This is different to what is seen in other insects where transferrin is expressed primarily in the fat body (Harizanova et al. 2005;Hirai et al. 2000;Yoshiga et al. 1999). This suggests that the accessory (milk) gland in tsetse may have taken up the role of the fat body in the synthesis of this protein. Its abundant presence in the milk ducts indicates that it is transferred into the intrauterine larvae in the mother's "milk" to nurture its development. The presence of transferrin protein in the hemolymph and oocytes, suggests that the protein synthesized in the milk gland is also secreted into the hemolymph. This would explain the areas of intense staining that are observed at the tips of the milk gland tubules in the immunohistochemical analysis. The presence of GmmTsf in the oocytes suggests that it is being taken up as a vitellogenic protein. The predominant yolk protein, GmmYP1 is expressed exclusively by the ovaries (Attardo *et al.*, 2006). The *GmmTsf* gene differs in that it is expressed predominantly in the fat body/milk gland tissues with significantly lower levels expressed in the ovaries (Fig. 1A Lanes 2+3). One possible way

that transferrin is entering the reproductive tract is via milk secretions. If milk spills into the ovaries from the uterus, this would put GmmTsf in proximity with the oocytes and allow the protein to be taken up by receptor mediated endocytosis. Another possibility is that the low levels of *GmmTsf* transcript generated in the ovary are enough to supply adequate GmmTsf protein to the developing oocytes.

In tsetse, transferrin is expressed in both sexes although its spatial and temporal pattern differs. In females the majority of expression is in the fat body/milk gland tissues whereas in males the gene is expressed in the reproductive tract (Strickler-Dinglasan et al. 2006). GmmTsf protein can be detected in the hemolymph and reproductive tracts of both males and females. The fact that the protein is found in the reproductive tract of both sexes suggests that this protein plays an important role in reproduction and/or development.

The mechanism of how GmmTsf gets into the male hemolymph from the reproductive tract is not yet known; however microscopic examination of the male reproductive tract has shown that the male has two large glandular organs attached to the reproductive tract that appear physiologically similar to the milk gland in the female (data not shown). These glands may be male analogues of the milk gland that are used for protein production and secretion. Further research will be needed to confirm these hypotheses.

The temporal expression of transferrin in tsetse also differs from that seen in other insects. In tsetse *GmmTsf* is only significantly expressed in the adult stages of development. This differs from *Drosophila* and *Aedes aegypti* where transferrin is expressed in the late larval, pupal and adult stages (Harizanova et al. 2005;Yoshiga et al. 1999). The differences in the expression pattern of transferrin between tsetse and other dipterans may be a result of the fact that the immature stages in tsetse (larvae and pupae) rely on their mother's nutritional resources for their development. The presence of GmmTsf in the ovaries and in the milk provided to the larvae suggests that it is playing an important role in the development of these stages. Interestingly, lactoferrin (a member of the transferrin family) is commonly found in high concentrations in mammalian milk secretions with human colostrum containing up to 7 grams per liter (Farnaud and Evans 2003). The presence of transferrin in milk secretions suggests functional conservation for this aspect of reproductive biology.

Multiple functions have been attributed to transferrin, such as iron binding/transport, immune peptide and vitellogenic protein (Nichol et al. 2002). In vertebrates transferrin has also been associated with induction of neutrophilic end-stage maturation, activation of enzymes regulating cell growth in conjunction with an insulin like growth factor and upregulation of chemokine synthesis by human proximal tubular epithelial cells (Evans et al. 1989;Tang et al. 2002;Wang et al. 1995). If these types of functions are also conserved in insects then they have the potential to be important for oogenesis, larvigenesis and metamorphosis. Further analysis of these functions will be important to determine the developmental role of GmmTsf in tsetse.

High levels of GmmTsf in the hemolymph of males and females suggest that the protein is also playing an important role in adults. Transferrins are know to function as iron transporters in blood feeding insects, moving iron from the digested blood meal through the hemolymph to target tissues for storage in ferritin protein complexes (Huebers *et al*., 1988). In a recent microarray based global expression analysis study in tsetse, we have observed significantly higher levels of transferrin expression in females in agreement with our analysis here (Attardo and Aksoy, unpublished observations). In these same experiments, the *Drosophila* NRAMP2 (DMT1) homolog is also detected as a highly expressed female specific gene. NRAMP2 in vertebrates has been shown to transport iron with high affinity in the intestines (Mackenzie and Hediger 2004). The potential interaction of transferrin with NRAMP2 and the role of this interaction in iron metabolism will require further investigation.

Transferrin has also been shown to be an important part of the immune system in insects and vertebrates. Upregulation of its transcription following immune challenge has been observed in a number of insects including *Ae. aegypti*, *Bombyx mori* and *Drosophila* (Yoshiga et al. 1999;Yoshiga et al. 1997;Yun et al. 1999) and also in the studies reported here in tsetse. Recent work on transferrin expression in goldfish (*Carassius auratus*) has shown that transferrin acts to activate the macrophage antimicrobial response (Stafford and Belosevic 2003). Specifically it results in the generation of the free radical nitrous oxide, which in turn is used to kill invading microbes. It is possible that in insects, transferrin is similarly acting upon the blood cells, hemocytes similar to vertebrate macrophages, in order to induce host immune responses. It is interesting that in the goldfish system transferrin was only active after proteolytic cleavage at a specific site. Proteolytic cleavage of transferrins has also been observed in *D, melanogaster* and *Ae. aegypti* (Harizanova et. al., 2005). Alignment of multiple insect transferrins (including tsetse) reveals conservation of the proposed cleavage site that was identified in the goldfish transferrin gene. Cleavage of transferrin appears to occur in *Glossina* as well, as multiple transferrin bands can be detected in the larval and pupal developmental stages. Whether transferrin is being cleaved to regulate development, immunity or both in the immature stages is not known and will require further study. Transferrin cleavage in immune challenged flies also has not yet been examined and requires further study to see if this mechanism may play a role in activating the host's antimicrobial defenses.

Another point of interest is that tsetse flies with a stable midgut trypanosome infection express lower levels of transferrin transcripts than uninfected flies. Furthermore, flies that were challenged but resisted trypanosome infection have close to normal levels of transferrin transcripts. This suggests the possibility that the trypanosomes in the infected flies might be influencing host gene expression in order to provide more hospitable living conditions, such as greater iron availability and lower levels of free radicals. Such a strategy would provide additional nutritional resources and security to the replicating parasites, but as a result undermine the host's reproductive fitness.

In conclusion, transferrin apparently plays a complex role in the life history of the tsetse fly. It appears to be involved in reproduction, development, homeostasis and immunity. Further analysis of this factor will yield important insights into the biology of the tsetse fly and the role of transferrin in all animals.

Acknowledgements

This research was supported by grants from the NIH AI51584 to Serap Aksoy and the NIH Ruth Kirschtein Postdoctoral Training Award F32 GM077964 to Geoffrey M. Attardo.

References

Aksoy S. Tsetse - A haven for microorganisms. Parasitology Today 2000;16(3):114–118. [PubMed: 10689331]

Attardo G, Strickler-Dinglasan P, Perkin S, Caler E, Bonaldo M, Soares M, El-Sayeed N, Aksoy S. Analysis of fat body transcriptome from the adult tsetse fly, *Glossina morsitans morsitans*. Insect Mol Biology 2006a;15(4):411–424.

Attardo GM, Guz N, Strickler-Dinglasan P, Aksoy S. Molecular aspects of viviparous reproductive biology of the tsetse fly (*Glossina morsitans morsitans*): Regulation of yolk and milk gland protein synthesis. Journal Insect Physiology 2006b;52:1128–1136.

Barrett M. The fall and rise of sleeping sickness. Lancet 1999;353:1113–1114. [PubMed: 10209970]

Dunkov B, Georgieva T. Insect iron binding proteins: Insights from the genomes. Insect Biochemistry and Molecular Biology 2006;36(4):300–309. [PubMed: 16551544]

Ekwanzala M, Pepin J, Khonde N, Moliho S, Bruneel H, De Wals P. In the heart of darkness:sleeping sickness in Zaire. Lancet 1996;348:1427–1430. [PubMed: 8937285]

Evans WH, Wilson SM, Bednarek JM, Peterson EA, Knight RD, Mage MG, McHugh L. Evidence for a factor in normal human serum that induces human neutrophilic granulocyte end-stage maturation in vitro. Leukemia Research 1989;13(8):673–682. [PubMed: 2477645]

Farnaud S, Evans RW. Lactoferrin--a multifunctional protein with antimicrobial properties. Molecular Immunology 2003;40(7):395–405. [PubMed: 14568385]

Harizanova N, Georgieva T, Dunkov BC, Yoshiga T, Law JH. *Aedes aegypti* transferrin. Gene structure, expression pattern, and regulation. Insect Molecular Biology 2005;14(1):79–88. [PubMed: 15663777]

Hirai M, Watanabe D, Chinzei Y. A juvenile hormone-repressible transferrin-like protein from the bean bug, *Riptortus clavatus*: cDNA sequence analysis and protein identification during diapause and vitellogenesis. Archives of Insect Biochemistry and Physiology 2000;44(1):17–26. [PubMed: 10790182]

Jamroz RC, Gasdaska JR, Bradfield JY, Law JH. Transferrin in a cockroach: molecular cloning, characterization, and suppression by juvenile hormone. Proceedings of the National Academy of Sciences of the United States of America 1993;90(4):1320–1324. [PubMed: 7679500]

Mackenzie B, Hediger MA. SLC11 family of H+-coupled metal-ion transporters NRAMP1 and DMT1. Pflugers Arch 2004;447(5):571–579. [PubMed: 14530973]

Moloo SK. An artificial feeding technique for *Glossina*. Parasitology 1971;63(3):507–512. [PubMed: 5139030]

Nichol H, Law JH, Winzerling JJ. Iron metabolism in insects. Annual Review Entomology 2002;47:535–559.

Park JH, Attardo GM, Hansen IA, Raikhel AS. GATA factor translation is the final downstream step in the amino acid/target-of-rapamycin-mediated vitellogenin gene expression in the anautogenous mosquito *Aedes aegypti*. The Journal of Biological Chemistry 2006;281(16):11167–11176. [PubMed: 16490782]

Sambrook, J.; Russell, DW. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Press; 2001.

Sillitoe RV, Hawkes R. Whole-mount immunohistochemistry: a high-throughput screen for patterning defects in the mouse cerebellum. Journal Histochemical Cytochemistry 2002;50(2):235–244.

Stafford JL, Belosevic M. Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. Developmental and Comparative Immunology 2003;27(67):539–554. [PubMed: 12697311]

Strickler-Dinglasan PM, Guz N, Attardo G, Aksoy S. Molecular Characterization of Iron Binding Proteins from *Glossina morsitans morsitans* (Diptera: Glossinidae). Insect Biochemistry and Molecular Biology 2006;36:921–933. [PubMed: 17098167]

Tang S, Leung JC, Tsang AW, Lan HY, Chan TM, Lai KN. Transferrin up-regulates chemokine synthesis by human proximal tubular epithelial cells: implication on mechanism of tubuloglomerular communication in glomerulopathic proteinura. Kidney International 2002;61(5):1655–1665. [PubMed: 11967015]

Wang LG, Liu XM, Wikiel H, Bloch A. Activation of casein kinase II in ML-1 human myeloblastic leukemia cells requires IGF-1 and transferrin. Journal of Leukocyte Biology 1995;57(2):332–334. [PubMed: 7852847]

Yoshiga T, Georgieva T, Dunkov BC, Harizanova N, Ralchev K, Law JH. *Drosophila melanogaster* transferrin. Cloning, deduced protein sequence, expression during the life cycle, gene localization and up-regulation on bacterial infection. European Journal of Biochemistry / FEBS 1999;260(2):414–420. [PubMed: 10095776]

Yoshiga T, Hernandez VP, Fallon AM, Law JH. Mosquito transferrin, an acute-phase protein that is up-regulated upon infection. Proceedings of the National Academy of Sciences of the United States of America 1997;94(23):12337–12342. [PubMed: 9356450]

Yun EY, Kang SW, Hwang JS, Goo TW, Kim SH, Jin BR, Kwon OY, Kim KY. Molecular cloning and characterization of a cDNA encoding a transferrin homolog from *Bombyx mori*. Biological Chemistry 1999;380(12):1455–1459. [PubMed: 10661875]

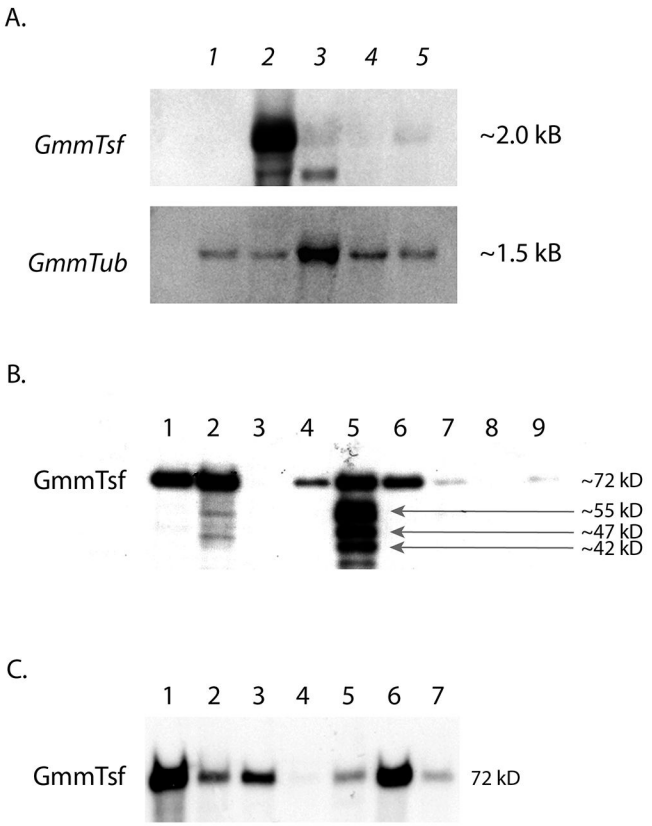

Fig 1. Tissue specificity of transferrin mRNA and protein expression

A. Northern blot analysis of tissue specific expression. Lanes: 1: midgut, 2: fat body and milk gland, 3: reproductive tract, 4: carcass, 5: larvae. Five μg of total RNA from each tissue sample was loaded per lane and the blot was hybridized to transferrin and control tubulin cDNA sequences

B. Tissue specific expression of transferrin protein. Lanes: 1: female carcass, 2: female fat body and milk gland, 3: female digestive tract, 4: female reproductive tract, 5: larvae, 6: male carcass, 7: male fat body, 8: male digestive tract, 9: male reproductive tract. Fly tissues were homogenized in equal volumes of buffer and homogenates from three flies were pooled for each sample. Each lane was loaded with the equivalent of 0.625% of the total protein from each sample.

C. Reproductive tract specific western blot of transferrin protein. Lanes: 1: female hemolymph, 2: uterus, 3: spermatheca, 4: inactive ovary, 5: active ovary, 6: male hemolymph, 7: testes. Fly tissues were homogenized in equal volumes of cracking buffer and homogenates from five flies were pooled for each sample. Each lane was loaded with a protein amount equivalent to that of the tissue of one fly.

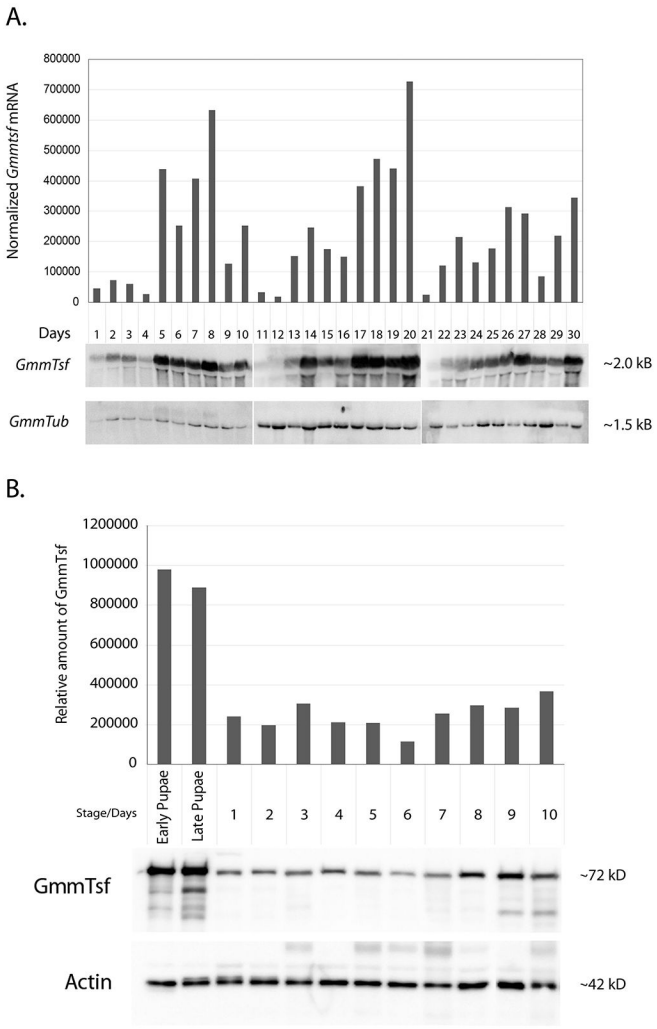

Fig 2. Time course analysis of *GmmTsf* mRNA and protein expression

A. Northern blot analysis of *GmmTsf* levels over time in days post eclosion. Each time point in the course is equal to 10 μg of pooled total RNA collected from 3 individual flies for that time point. *GmmTub* was used as a loading control. Data from the Northern blots was quantitated by phosphoimager and normalized against *GmmTub* levels. Data points on the bar graphs represent the average level of expression over a 3-day period. Data points used to generate the graph are shown in the Northern blot image below the graph.

B. Western blot analysis of GmmTsf levels over time in days post eclosion. Each time point in the course is equal to 10 μg of total protein collected from three individual flies for that time point. Blots were probed with polyclonal antisera specific to GmmTsf. Antibodies against *Drosophila* Actin were used as a loading control.

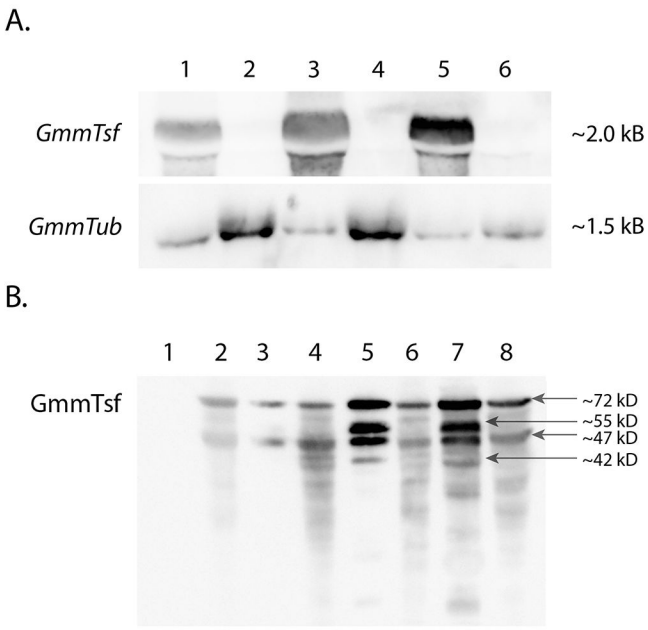

Fig 3. Transferrin transfer dynamics between mother and intrauterine offspring

A. Northern blot analysis of *GmmTsf* expression in mothers versus offspring. Lanes: 1: mother of 1st instar larvae, 2: 1st instar larvae, 3: mother of 2nd instar larvae, 4: 2nd instar larvae, 5: mother of 3rd instar larvae, 6: 3rd instar larvae. Five μg of total RNA from each tissue sample was loaded per lane and the blot was hybridized with DIG labeled probes generated from transferrin and tubulin cDNA sequences

B. Western blot analysis of GmmTsf in mothers versus offspring. Lanes: 1: embryo, 2: mother of embryo, 3: 1st instar larvae, 4: mother of 1st instar larvae, 5: 2nd instar larvae, 6: mother of 2nd instar larvae, 7: 3rd instar larvae, 8: mother of 3rd instar larvae. Either individual whole flies or their larvae were homogenized in equal volumes of cracking buffer. Each lane was loaded with the equivalent of 0.625% of the total protein from each sample

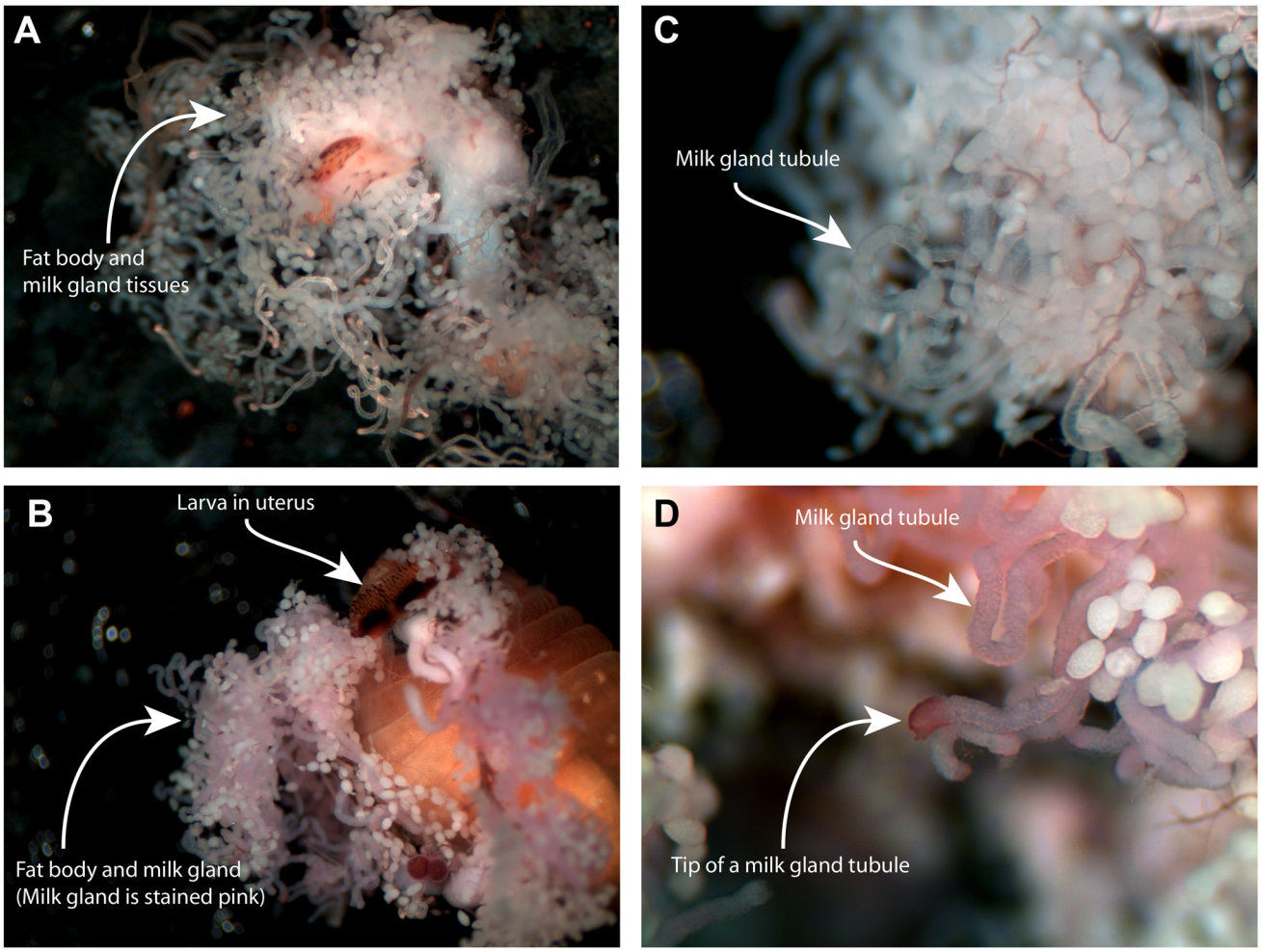

Fig 4. Localization of GmmTsf by immunohistochemistry

Milk gland/fat body and reproductive tissues were dissected from pregnant flies carrying 2nd or 3rd instar larvae. Tissues were fixed in paraformaldehyde and stained with either preimmune serum (negative controls A + C) or antiserum generated against recombinant transferrin as primary antibodies (B + D). Tissues were stained with vector red dye and photographed under a dissecting scope.

A. View of milk gland and fat body tissue (negative control).

B. View of milk gland and fat body tissue (stained with transferrin antibody)

C. Close up view of milk gland and fat body tissue (negative control).

D. Close up view of milk gland and fat body tissue (stained with transferrin antibody)

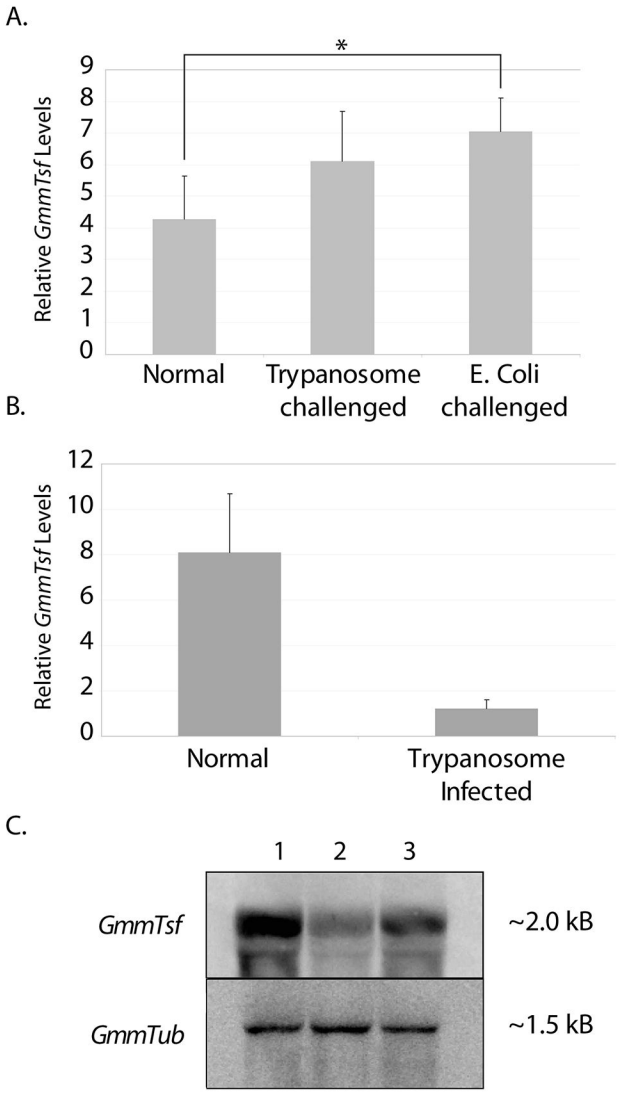

Fig 5. Expression of *GmmTsf* following immune challenge

A. qRT-PCR expression analysis of GmmTsf 24 h post immune challenge with Ytat1.1 trypanosomes and E. coli normalized against the housekeeping gene tsetse tubulin (GmmTub). (The * represents the difference between the normal sample and the E. coli sample as statistically significant with a t-test score of <0.05.)

B. qRT-PCR expression of GmmTsf from Ytat1.1 infected and uninfected flies normalized against GmmTub.

C. Northern analysis of GmmTsf from normal flies (lane 1), Ytat1.1 infected (lane 2) and self-cured flies (lane 3).