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The *Glossina* proteolytic lectin (*Gpl*) gene is expressed only in members of *Glossina* species

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Abstract. Differentiation of bloodstream-form trypanosomes into procyclics in tsetse flies (Diptera: Glossinidae) is a crucial step in the establishment of midgut infections. A number of factors have been implicated in the transformation process, including enzymes and lectins or lectin-like molecules. Recently, *Glossina* proteolytic lectin (*Gpl*) gene, which encodes a protein with both lectin and trypsin activities has been shown to stimulate transformation of bloodstream-form trypanosomes into procyclics *in vitro*. Using RT-PCR, we show that the induction of *Gpl* gene expression by blood meal occurs only in *Glossina fuscipes fuscipes* Newstead, *Glossina austeni* Newstead, *Glossina pallidipes* Austen, and not in the *Anopheles gambiae* Giles *sensu stricto*, *Phlebotomus duboscqi* Neveu-Lemaire, *Rhipicephalus appendiculatus* Neumann and *Stomoxys calcitrans* (Linnaeus). The expression means of *Gpl* mRNA in *G. f. fuscipes* following a blood meal were significant ($P < 0.05$) with low expression in teneral flies and reaching a maximum between 48 and 72 h ($P < 0.05$), suggesting time-dependent regulation of the transcription. The expression of the *Gpl* gene was significantly lower ($P < 0.05$) in *G. f. fuscipes* fed on blood meal infected with *Trypanosoma brucei brucei* as compared with *G. f. fuscipes* fed on uninfected blood meal. This suggests some form of interaction of *T. b. brucei* or the parasite products with *Gpl* within the tsetse midgut leading to down-regulation of the *Gpl* gene. Additionally, refractory *G. f. fuscipes* expressed higher ($P < 0.05$) transcript abundance than the susceptible *G. pallidipes*.

Key words: *Glossina* proteolytic lectin gene, midgut, bloodmeal, *Glossina* species, trypanosome

Introduction

The transformation of bloodstream-form trypanosomes into the procyclic or midgut form within the tsetse fly midgut is a crucial first step in the establishment of midgut infection (Roditi and Pearson, 1990; Maudlin, 1991). Several factors, including the source of blood (Nguu *et al.*, 1996), a shift in blood meal temperature from 37 °C in the vertebrate host to 27 °C in the vector (Cross and

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Manning, 1973; Bienen *et al.*, 1981), supplementation of *cis*-aconitate (Brun and Schonenberger, 1981), mild acidic conditions in the gut of the vector (Rolin *et al.*, 1998) and trypsin (Imbuga *et al.*, 1992; Hunt *et al.*, 1994), have been implicated in the process. While all these factors appear to stimulate trypanosome transformation to the procyclic form *in vitro*, this process occurs much faster *in vivo* in the tsetse midgut.

Ibrahim *et al.* (1984) first reported the presence of a glucosamine-binding lectin in tsetse midgut. Subsequent studies by Imbuga *et al.* (1992) showed that *Glossina morsitans morsitans* (Westwood) (Diptera: Glossinidae) midgut extract was capable of stimulating transformation of bloodstream *Trypanosoma brucei brucei* (Trypanosomatidae). Furthermore, the midgut homogenates could induce transformation only in the presence of blood. This finding indicated that the efficient transforming activity was associated with the tsetse midgut. Interestingly, a blood meal-induced molecule with lectin and trypsin activities from midguts of tsetse was later isolated and characterized (Osir *et al.*, 1995). Experiments using antibodies against procyclin provided direct evidence for its involvement in triggering transformation of bloodstream trypanosomes *in vitro* (Abubakar *et al.*, 2003). This induction proceeded more efficiently at optimal lectin–trypsin concentrations, similar to those present in the first 24 h after the blood meal. Inhibition of the activity of the molecule by D-glucosamine significantly blocked the lectin–trypsin complex-induced transformation of bloodstream-form trypanosomes (Abubakar *et al.*, 2003). Despite these findings, it remained unclear whether trypsin activity alone is a sufficient signal for parasites to be committed to transform, or whether lectin activity in the midgut has to come into play.

Recent studies on immune-responsive genes showed that the tsetse immune system could discriminate not only between molecular signals specific for bacteria and trypanosome infections but also between different life stages of trypanosomes (Hao *et al.*, 2001). However, relatively little information is available about the genes involved in the transformation process despite the critical role it plays in transmission of trypanosome infection. Recently, a gene encoding for a proteolytic lectin, with trypanosome-transforming activity, was isolated from a midgut cDNA library of *G. f. fuscipes* Newstead and expressed in bacteria (Abubakar *et al.*, 2006). The sequence, designated *Glossina* proteolytic lectin (*Gpl*), contained the catalytic domain of serine protease with aspartate at the specificity pocket, suggesting a typical trypsin. A similar gene was identified from another tsetse fly, *Glossina austeni* Newstead and expressed in both baculovirus and bacteria systems (Amin *et al.*, 2006). Interestingly, both baculovirus and bacterium expressed *Gpl* showed no significant differences in their activities, indicating that *Gpl* does not require a carbohydrate moiety for enzymatic or trypanosome-transforming activities (Amin *et al.*, 2006). Although the important role played by the blood meal-induced *Gpl* in transmission of trypanosome infection has been ascertained, it is presently unclear whether *Gpl* is confined within members of *Glossina* species, or is expressed in other haematophagous arthropods. Likewise, our knowledge about the gene expression profile upon blood meal acquisition and its regulation is limited. The present study was, therefore, undertaken to gain further insight into the expression pattern of the *Gpl* gene.

Here, we report on the expression of *Gpl* gene, in laboratory reared *G. fuscipes fuscipes* Newstead, *Glossina pallidipes* Austen, *G. austeni* Newstead (Diptera: Glossinidae) and its absence in other haematophagous arthropods: *Stomoxys calcitrans* (Linnaeus) (Diptera: Muscidae), *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae), *Phlebotomus duboscqi* Neveu-Lemaire (Diptera: Psychodidae) and *Rhipicephalus appendiculatus* Neumann (Acari: Ixodidae). The regulation and expression levels of the *Gpl* gene following infective and non-infective blood meal are also presented.

Materials and methods

Experimental arthropods and parasites

All experimental arthropods used in the experiments were 24 h post-emergence except *S. calcitrans* and *A. gambiae* s.s., which were field collected and 5 days old, respectively. *Glossina f. fuscipes*, *G. austeni* and *G. pallidipes* (both males and females) were obtained from the Animal Rearing and Containment Unit (ARCU) of ICIPE, Nairobi, Kenya. They were maintained at 24–27 °C with 55% humidity. Five-day-old female *A. gambiae* s.s. (Ifakara strain) were obtained from ARCU colony mosquitoes. *Phlebotomus duboscqi* were obtained from a colony that originated from sandflies collected in animal burrows and from termite hill ventilation shafts in Rabai area near the town of Marigat in Baringo district, Rift Valley province in Kenya. This colony has since been established and maintained at 28 °C with 80% humidity at the Kenya Medical Research Institute's insectaries (Beach *et al.*, 1986). *Rhipicephalus appendiculatus* were obtained from ARCU ticks colony and maintained at 28 °C with a relative humidity of 85%. Wild *S. calcitrans* were obtained from Marurui animal farm (Nairobi, Kenya). *Trypanosoma b. brucei* strain 3397 was obtained from the Trypanosomiasis Research Centre, Nairobi, Kenya.

Sample preparation

To screen for the expression of *Gpl* in haematophagous arthropods, *A. gambiae s.s.* were fed once on mouse blood, *P. duboscqi* were fed once on anaesthetized hamster, *R. appendiculatus* were fed once on rabbit blood, *S. calcitrans*, *G. f. fuscipes* and *G. austeni* were fed once on whole pig blood using an artificial membrane system and sampled after 72 h.

To study expression profiles of *Gpl*, 200 teneral *G. f. fuscipes* adults were fed once on whole pig blood using an artificial membrane system. Engorged flies were separated and put in cages. Six flies were sampled at 1, 6, 12, 24, 36, 48, 72, 96 and 120 h post-feeding and used for gene expression analysis at each target time point.

To study expression of *Gpl* following an infective and non-infective blood meal, 50 teneral adults of *G. f. fuscipes* were fed once on an uninfected anaesthetized Wistar rat. Engorged flies were separated in cages, and four flies were sampled at 24, 48 and 72 h post-feeding. A second group of 200 *G. f. fuscipes* was fed on an anaesthetized Wistar rat, infected with *T. b. brucei* and sampled at 24, 48 and 72 h post-feeding.

To compare expression levels of *Gpl* between *Glossina* species, 30 *G. f. fuscipes* and 30 *G. pallidipes* were fed on an uninfected Wistar rat. Engorged flies were separated in cages, and four flies were sampled at 24, 48 and 72 h post-feeding.

The midguts of all experimental arthropods were carefully dissected, washed several times in RNase-free PBS [0.1 M sodium phosphate (pH 8.0)] containing 0.15 M sodium chloride to remove any haemolymph.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using RNAgents® Total RNA Isolation System (Promega, Madison, WI, USA) and reverse transcribed using SMART™ PCR cDNA Synthesis Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) according to manufacturer's instructions.

Expression of Gpl *gene in various haematophagous arthropods*

Assessment of *Gpl* gene induction by blood meal in haematophagous arthropods was carried out using RT-PCR. The cDNAs of *A. gambiae* s.s., *S. calcitrans*, *P. duboscqi*, *R. appendiculatus*, *G. austeni* and *G. f. fuscipes* were amplified using *Gpl*-specific primers (*Gpl*, F: 5′-TTT GGA TCC ATG AAG TTT GCA GTG TTC GC-3′ and *Gpl*, R: 5′-CGG TAG TAA GCT TAC AAA AGT TGC GCA TAG-3′). The thermocycling parameters were as follows: 1 cycle of 94 °C for 1 min; 39 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 8 min in a MJ Research PTC-100 thermocycler.

Determination of expression levels of Gpl *in* G. f. fuscipes

The levels of *Gpl* gene in *G. f. fuscipes* were determined by multiplex titration RT-PCR as described by Nebenfuhr and Lomax (1998). A 1:2 15-fold dilution was carried out on the original cDNA template at each time point. Amplification was carried out in a volume of 25 μl, which contained 0.05 mM dNTP, 1 unit of Taq polymerase, 2 mM $MgCl_2$ and 0.15 μg of *Gpl*-specific primers including glyceraldehyde phosphate dehydrogenase (GAPDH) gene primers (F: 5′-TAG ATT GGA CTG TGC GCT TG-3′ and R: 5′-AAA TGG GTG GAT GGT GAG AG-3′) as the internal control in a MJ Research PTC-100 thermocycler. The cycling parameters were as described in 'Expression of *Gpl* gene in various haematophagous arthropods'. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and visualized using UV transilluminator. Dilution step of the last visible band was noted.

Determination of the effect of trypanosomes on Gpl *gene expression*

Quantitative competitive RT-PCR (QC-RT-PCR) was used to determine the effect of trypanosomes on *Gpl* gene expression (Seibert and Larrick, 1992). First, midgut infection was ascertained by PCR using parasite specific α-tubulin primers (F: 5′-GCG TGA GCC TAT GTG CAT CCA CAT TGG TCA G-3′ and R: 5′-CTA GTA CTC CTC CAC ATC CTC CTC ACC GTC C-3′). Only infected midguts were included in the study. At most, four infected guts were used for each time point. The thermocycling parameters were 1 cycle of 94 °C for 2 min; 39 cycles of 94 °C for 30 sec, 60 °C for 1 min, 72 °C for 1.5 min and a final extension at 72 °C for 8 min.

To determine initial estimation of copy number of RNA transcripts, reverse transcription reactions were performed separately on an aliquot of RNA competitor/internal standard (*c.* 6.16×10^{13}) and on a standard amount of each sample RNA (300 ng) using SMART™ PCR cDNA Synthesis Kit (Clontech Laboratories, Inc.) and *Gpl* reverse primer, according to manufacturer's instructions. The competitor RNA consisted of the same target mRNA, synthesized with a deletion of *c.* 125 bases (Kephart, 1998). Competitor cDNA was then serially diluted 1:100 sixfold, and 2 μl sample DNA was spiked to the serially diluted competitor cDNA and co-amplified using *Gpl*-specific primers. The thermocycling parameters were as described in 'Expression of *Gpl* gene in various haematophagous arthropods' but

the amplification was terminated at the exponential phase (30 cycles). Amplification products were analysed using 2.0% agarose gel. Gel images were captured under UV transilluminator. The dilution of the competitor cDNA that yielded PCR products of bands of equal intensities as the target samples were used as bases for subsequent reactions.

To obtain more precise value of *Gpl* transcripts, stock competitor RNA (2 μl) was first diluted 1:100 sixfold. A further 1:2 eightfold dilution was done at the point where competitor and target cDNA gave bands of equal intensity. The 1:2 serially diluted cRNA was then co-reverse transcribed with 300 ng of total sample RNA using *Gpl*-specific reverse primer and resulting cDNAs co-amplified using *Gpl*-specific primers. The PCR products were separated in a 2.0% agarose gel. The gel images were captured under UV transilluminator and the intensities of the bands quantified using the NIH image software (Scion Image for Windows Beta 4.0.2). To determine the competition equivalence point, the logarithms of the band intensity ratio of target product to competitor product were plotted as a function of the logarithm of the initial amount of competitor RNA molecules added. The initial amount of the target mRNA transcripts in the sample was calculated by linear regression analysis. The copy numbers of *Gpl* transcripts were transformed into logarithmic scale and analysed using two-way ANOVA.

Statistical data analysis

The differences in the number of bands (for multiplex titration RT-PCR) visible under UV as a function of time were analysed by ANOVA using the GLM procedure of SAS (1999). The Student–Neuman–Keuls test separated means at different sampling times at $P < 0.05$.

Results

Expression of Gpl *gene in various haematophagous arthropods*

The screening of *Gpl* in haematophagous arthropods revealed that this gene was only expressed in *Glossina* species but not in the other haematophagous arthropods used in the study. In the unfed *Glossina* species, the transcript intensity was very little as compared with the fed *Glossina* guts (Fig. 1; lanes 9–12).

Expression levels of Gpl *in* G. f. fuscipes

Multiplex titration RT-PCR data showed significantly low levels of expression in teneral guts ($P < 0.05$; Fig. 2). The expression was rapidly induced after 1 h post-blood meal and peaked between 48 and 72 h. The transcript abundance then decreased from 96 h post-feeding. Further decrease was observed after 120 h post-feeding but the level did not return to levels close to those present in teneral guts (Fig. 2).

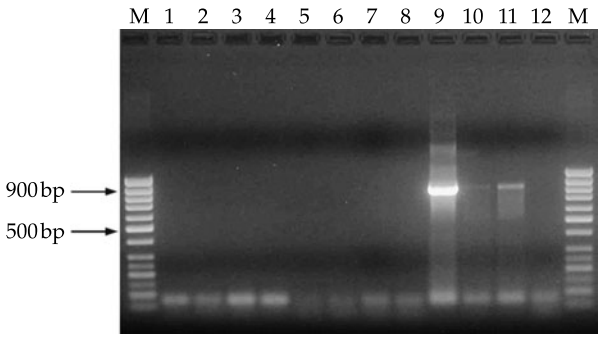

Fig. 1. Amplification of *Gpl* gene in various haematophagous arthropods. Lane M represents 50 bp DNA marker; 1 and 2, fed and unfed *Anopheles gambiae*; 3 and 4, fed and unfed *Rhipicephalus appendiculatus*; 5 and 6, fed and unfed *Phlebotomus duboscqi*; 7 and 8, fed and unfed *Stomoxys calcitrans*; 9 and 10, fed and unfed *Glossina fuscipes fuscipes*; 11 and 12, fed and unfed *Glossina austeni*. All unfed gut cDNAs were used as the controls

Effect of trypanosomes on Gpl *gene expression*

With QC-RT-PCR, the *Gpl* expression was parasite responsive, with expression being significantly lower in trypanosome infected *G. f. fuscipes* ($P < 0.05$; Fig. 3). At 24, 48 and 72 h, infected *G. f. fuscipes* had 6.02×10^5, 6.73×10^5 and 7.92×10^5, while uninfected had 7.29×10^5, 7.45×10^5 and 8.89×10^5 *Gpl* copy numbers, respectively.

Gpl *expression among* Glossina *groups*

Gpl gene copy numbers varied significantly between *G. pallidipes* and *G. f. fuscipes* ($P < 0.05$; Fig. 4). At 0, 24, 48 and 72 h, *G. pallidipes* had 4.82×10^5, 5.25×10^5, 6.39×10^5 and 7.19×10^5, while *G. f. fuscipes* had 6.22×10^5, 7.29×10^5, 7.45×10^5 and 8.89×10^5 *Gpl* copy numbers, respectively.

Discussion

Screening of *Gpl* gene expression in blood feeding arthropods showed that *Gpl* is expressed in members of *Glossina* species only. In this study, oligo dT primers, which results in reverse transcription of all possible pools of mRNA present within the total RNA were used during cDNA synthesis. *Gpl*-specific primers, however, only gave an amplification product in *Glossina* cDNA

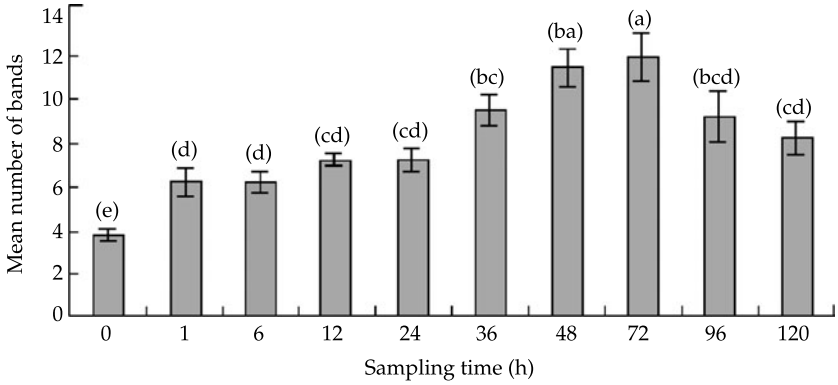

Fig. 2. Schematic analysis of *Gpl* transcripts expression in *Glossina fuscipes fuscipes* as a function of time in the blood meal digestion process. Each bar represents the mean number (±SEM) of visible bands obtained under UV using *Gpl*-specific primers at 0, 1, 6, 12, 24, 36, 48, 72, 96 and 120 h post-feeding. Means sharing the same letter are non-significantly different

suggesting that the rest of blood feeders lack the transcripts of interest. In addition, Basic Local Alignment Search Tool of *Gpl* sequence from both *G. austeni* and *G. f. fuscipes* did not match any of the haematophagous sequences apart from *S. calcitrans* midgut-specific serine protease 1 (Ssp1) mRNA, complete cds which gave a score of 4e-48 indicating lack of a conserved region of this gene in other haematophagous arthropods studied. This may suggest that this gene is unique to *Glossina* species and hence plays a role in tsetse–trypanosome interaction other than blood meal digestion within the midgut. It is tempting to speculate that the presence of *Gpl* in tsetse midgut and its absence in other haematophagous arthropods is the reason why tsetse flies are the only known cyclic vectors, while the others are implicated in mechanical transmission of trypanosomes. This finding agrees with an earlier report by Osir *et al.* (1995) where the antibodies to *Gpl* did not cross-react with midgut extracts of *P. duboscqi*, *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) and *S. calcitrans*.

Following a blood meal, the midgut digestive enzymes are secreted in response to increased protein content in the gut (Gooding, 1974) and their levels in the lumen change throughout the digestion process (Gooding, 1977). The regulation of expression for digestive enzymes varies widely in different insects. In *A. aegypti*, the synthesis of early trypsin appears to be regulated at translation level since early trypsin mRNA can be detected in unfed fly gut (Noriega *et al.*, 1996). Late trypsin is detectable after 8 h and reaches its maximum level after *c.* 24 h (Noriega *et al.*, 1994). However, insects with continuous digestive systems such as *Stomoxys* and *Glossina* store and dehydrate the blood in a specialized region of midgut (reservoir) before it is passed through the gut where different regions are responsible for digestion (opaque zone) and absorption (lipoid zone) activities. In *Stomoxys* spp., the regulation of digestive enzymes has been shown to be at the post-transcriptional level with protein levels reaching a maximum shortly after obtaining the blood meal (8–10 h) (Mofatt *et al.*, 1995).

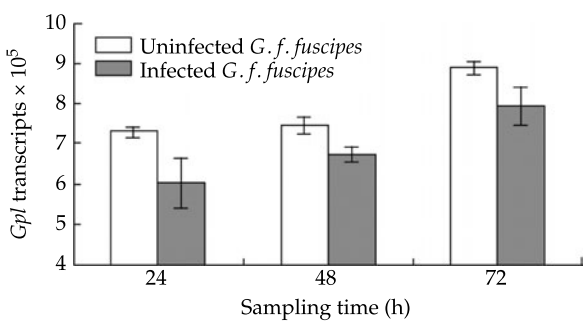

Fig. 3. Schematic analysis of *Gpl* transcripts expression in infected and uninfected *Glossina fuscipes fuscipes* as a function of time during bloodmeal digestion process. Each bar represents the mean copy numbers of *Gpl* transcripts of four independent experiments (±SEM) at 24, 48 and 72 h post-feeding. At each time point, the *Gpl* copy numbers were significantly different ($P < 0.05$)

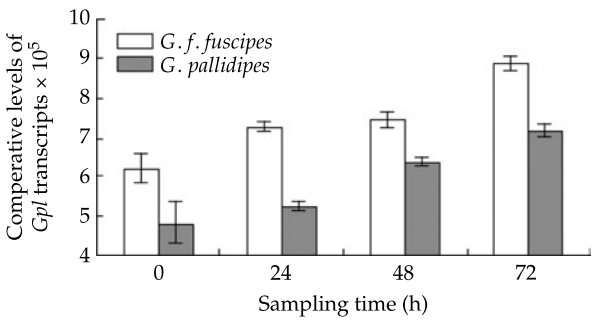

Fig. 4. Schematic analysis of *Gpl* transcripts expression in *Glossina fuscipes fuscipes* and *Glossina pallidipes* as a function of time in the bloodmeal digestion process. Each bar represents the mean copy numbers of *Gpl* transcripts of four independent experiments (±SEM) at 0, 24, 48 and 72 h post-feeding. At each time point, the *Gpl* copy numbers were significantly different ($P < 0.05$)

Analysis of expression of *Gpl* in *G. f. fuscipes* gut showed significant variation of the mRNA abundance throughout the digestion cycle. In teneral flies, low transcript abundance levels were observed and peaked between 48 and 72 h. At 120 h post-feeding, *Gpl* mRNA levels did not return to a level close to that present in teneral guts. This observation suggests that *Gpl* is blood meal induced and its regulation may be at the transcription level. A similar observation has been reported in other blood meal-induced midgut genes. Yan *et al.* (2002) observed very low levels of carboxypeptidase and metalloprotease gene transcripts in teneral *G. m. morsitans*, but the expression was rapidly induced as early as 1 h post-blood meal acquisition. These results suggest that teneral gut may indeed represent a unique physiological state. The low expression of some gut enzymes may be among the factors contributing to greater susceptibility to trypanosome infection at this early adult stage (reviewed in Welburn and Maudlin, 1999). Low levels of trypsin or trypsin-like enzymes in the gut of tsetse flies cleave off the trypanosome surface coat and this may provide the signal for the parasite to transform from bloodstream to midgut forms (Yabu and Takayanagi, 1988; Imbuga *et al.*, 1992; Van den Abbelle and Decleir, 1992; Osir *et al.*, 1993). On the other hand, high levels of these enzymes result in lysis of the parasite (Imbuga *et al.*, 1992; Van den Abbelle and Decleir, 1992).

While digestive functions of the gut tissue are central for insect viability, the gut is also the first site of interaction between tsetse and trypanosomes and stands to influence the disease outcome. The N-terminal domains of all procyclins and the surface coat of the procyclic trypanosome are quantitatively removed by proteolysis in the fly, but not in culture, indicating a close interaction between gut enzymes and parasites (Acosta-Serrano *et al.*, 2001). In tsetse, *Gpl* expression exhibits a parasite-responsive profile with trypanosome-infected flies having low levels of the transcripts. This down-regulation of *Gpl* gene may be one of the mechanisms used by the parasites to survive the hostile environment found in the midgut. Both trypanosomes and bacterial challenges are reported to down-regulate several midgut immune response genes, thereby creating an environment for midgut infections such as in *Drosophila* spp. (Diptera: Tephritidae) and *Manduca* spp. (Lepidoptera: Sphingidae) (Lehane *et al.*, 2003). On the other hand, trypanosome infection has been reported to up-regulate several tsetse midgut genes including cathepsin B, carboxypeptidase and peroxidases (Yan *et al.*, 2002; Lehane *et al.*, 2003). This may suggest that trypanosome parasites modulate expression of several tsetse midgut genes.

Variation of *Gpl* expression observed between *G. pallidipes* and *G. f. fuscipes* may be related to the greater refractoriness of *G. f. fuscipes* to trypanosome infection as compared with *G. pallidipes*. The two species belong to the *palpalis* and *morsitans* groups of tsetse, respectively, which differ in their vectorial capacities. Kongoro *et al.* (2002) reported a similar variation between the groups where the midgut trypsin activity levels were higher in *G. f. fuscipes* than in *G. morsitans* and *G. morsitans centralis*. Similarly, studies in *A. gambiae* revealed that the *Plasmodium* refractory traits express higher levels of serine protease gene *Ag Sp24D* than the susceptible mosquitoes (Han *et al.*, 1997).

However, the mechanism of lectin–trypanosome interaction in the tsetse fly seems to be complex. It is not known if the *Gpl* triggers transformation in all trypanosome species that have midgut stages since it has only been done in *T. b. brucei*. Further work is required to establish if *Gpl* expression in the midgut varies depending on the parasite species. Hence, additional characterization of the molecule is necessary in order to figure out how effective vector control strategy can be based on this proteolytic lectin.

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