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Nutritional stress affects the tsetse fly's immune gene expression

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Abstract. Tsetse-transmitted trypanosomiasis poses a serious threat to human and animal health in sub-Saharan Africa. The majority of tsetse flies (*Glossina* spp.) in a natural population will not develop a mature infection of either *Trypanosoma congolense* or *Trypanosoma brucei* sp. because of refractoriness, a phenomenon that is affected by different factors, including the tsetse fly's immune defence. Starvation of tsetse flies significantly increases their susceptibility to the establishment of a trypanosome infection. This paper reports the effects of nutritional stress (starvation) on (a) uninduced baseline levels of gene expression of the antimicrobial peptides attacin, defensin and cecropin in the tsetse fly, and (b) levels of expression induced in response to bacterial (*Escherichia coli*) or trypanosomal challenge. In newly emerged, unfed tsetse flies, starvation significantly lowers baseline levels of antimicrobial peptide gene expression, especially for attacin and cecropin. In response to trypanosome challenge, only non-starved older flies showed a significant increase in antimicrobial peptide gene expression within 5 days of ingestion of a trypanosome-containing bloodmeal, especially with *T. brucei* bloodstream forms. These data suggest that a decreased expression of immune genes in newly hatched flies or a lack of immune responsiveness to trypanosomes in older flies, both occurring as a result of fly starvation, may be among the factors contributing to the increased susceptibility of nutritionally stressed tsetse flies to trypanosome infection.

Key words. *Glossina morsitans*, *Trypanosoma brucei brucei*, *Trypanosoma congolense*, antimicrobial peptides, infection, starvation.

Introduction

Tsetse flies (Diptera: Glossinidae) are obligate blood-feeding insects that cyclically transmit African trypanosomes, protozoan flagellated parasites that cause nagana in livestock and sleeping sickness in humans. The developmental cycle of the *Trypanosoma congolense* or *Trypanosoma brucei brucei* parasite in the insect vector starts when the tsetse fly feeds on a trypanosome-infected mammalian host. From this point on, the ingested parasite must undergo a series of developmental stages that are located in the alimentary tract and the mouthparts or salivary glands of the fly. This complex journey within the tsetse fly infers two main barriers which the parasite must overcome: (a) the establishment of a procyclic infection in the tsetse midgut within 3–5 days following the infective bloodmeal, and (b) migration and differentiation to the final metacyclic infectious forms in the mouthparts or salivary glands, depending on trypanosome species (Leak, 1999; Van Den Abbeele *et al*., 1999). These developmental barriers are evidenced by the low proportion of trypanosome infections in the midgut of experimentally infected flies and the fact that only a limited proportion of these midgut infections will finally give

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rise to a mature infection. This implies that only a small proportion of flies (both males and females) can eventually transmit the disease, a phenomenon referred to as 'refractoriness'. Our understanding of the tsetse-trypanosome molecular interactions underlying this refractoriness is still limited. Many factors, including midgut lectins, antioxidants and symbiotic associations in the tsetse fly, have been suggested to affect the success or failure of the parasite's development (Aksoy *et al*., 2003; Munks *et al*., 2005; Abubakar *et al*., 2006; Macleod *et al*., 2007). In addition, the insect immune system has been shown to play an important role in determining the fate of a trypanosome infection (Hao *et al*., 2001). Indeed, tsetse flies are able to synthesize a range of antimicrobial peptides (AMPs), such as attacin, defensin, cecropin and diptericin, in response to the presence of a 'foreign' micro-organism. This tsetse innate immune response is suggested to affect the establishment and maturation of trypanosomes in the vector (Hao *et al*., 2001; Boulanger *et al*., 2002; Lehane *et al*., 2004; Attardo *et al*., 2006; Hu & Aksoy, 2006). Moreover, Lehane *et al*. (2008) provided evidence that an immune-responsive transferrin is also involved in the tsetse-trypanosome interaction.

The proportions of infected flies in a tsetse population, as well as age-specific patterns of susceptibility, are important factors that affect the epidemiology of trypanosomiasis. In principle, the large majority of infected tsetse flies are considered to have acquired the infection at their first bloodmeal as a teneral fly (newly emerged unfed fly). Older flies are reported to be refractory to trypanosome infection and to contribute little to the overall infection rate of a tsetse population. However, under specific physiological conditions, both teneral and older flies can become more susceptible to trypanosome infection. Indeed, starving teneral and 20-day-old tsetse flies for several days resulted in significant increases in both *T. congolense* and *T. brucei* spp. infections (Gingrich *et al*., 1982; Kubi *et al*., 2006). The underlying mechanism that causes this important change in susceptibility has not yet been clarified. Therefore, in this study, we evaluated whether starvation of the tsetse fly affects uninduced and pathogen-induced innate immune responses in a way that may contribute to the increased susceptibility of starved tsetse flies to trypanosome infection. As a limited experimental read-out for the fly fat body-regulated immune response, the gene expression levels of the AMPs attacin, defensin and cecropin were measured. These are AMPs regulated by the immune deficiency (IMD) signalling pathway that are suggested to interfere with trypanosome development in the tsetse fly (Hu & Aksoy, 2006).

Materials and methods

Tsetse flies

Male *Glossina morsitans morsitans* Westwood tsetse flies from the colony maintained at the Institute of Tropical Medicine (Antwerp, Belgium) were used throughout the experiments. The origin of this tsetse fly colony and the rearing conditions were described by Elsen *et al*. (1993).

Only male flies were used in the experimental set-up because an accurate fat analysis of female tsetse flies is hampered by the presence of a developing larva in the female abdomen as a result of the tsetse's viviparous reproduction mode. Moreover, the molecular interactions that play a role in trypanosome development in the tsetse fly are assumed to be similar in male and female flies; thus male flies can be used as an experimental model to study these interactions and the factors affecting them.

Nutritional stress by starvation

In all experiments, four groups of flies were compared. The groups differed in age and nutritional status. Group 1 comprised non-starved teneral flies (= newly emerged unfed flies) aged < 32 h (TF0). Group 2 included teneral flies that had been starved for 4 days (TF4). Group 3 consisted of 20-day-old flies starved for 2 days after their last bloodmeal (AD2). Group 4 represented 20-day-old flies starved for 7 days after their last bloodmeal (AD7).

Fat content determination

A total of 96 flies (24 flies per group) were used in this set of experiments. Flies were killed at -20°C. Legs and wings were removed and carcasses were dried at 80°C to constant weight. To increase the precision of the measurement of dry weights, flies were pooled by three for weighing. Lipids were extracted using chloroform for 3 days (Langley *et al*., 1990). Chloroform was replaced daily. After fat extraction and drying to constant weight, the residual dry weight of the flies was determined. The fat content of the flies was calculated as the difference between the dry and residual weights. All weightings were performed on a Sartorius® analytical balance (Sartorius AG, Göttingen, Germany) ($\pm$ 0.1 mg).

Bacterial and trypanosomal challenge of tsetse flies

The four experimental groups of flies were immune-challenged by injecting bacteria or by feeding the flies with trypanosome-infected blood.

To evaluate the bacteria-induced immune response in each of the experimental groups, batches of 40 flies for each group were micro-injected either with 2 μL phosphate buffered saline (PBS) (control) or with 2 μL of a suspension of live *Escherichia coli* (DH10B strain) (OD_{620}: approximately 0.6). Four days after the bacterial injection, whole abdomens were removed and pooled by two for total RNA extraction.

For the trypanosomal challenge, batches of 40 flies for each group were given a bloodmeal on anaesthetized mice (strain NMRI) showing a parasitaemia of approximately $10^{8.1}$ trypanosomes/mL blood. The trypanosome strains used were *T. congolense* IL1180, a strain originating from Serengeti in Tanzania (Geigy & Kauffman, 1973) and *T. b. brucei* ANTAR 1, a strain derived from the stock EATRO 1125 (Le Ray *et al*., 1977). Control flies were given a bloodmeal on uninfected mice. Whole abdomens were removed from the infected flies on days 1, 3 or 5 following the initial bloodmeal and pooled by two for total RNA extraction.

RNA extraction and quantification

Immediately after removal, the abdominal tissue was manually homogenized with a Teflon pestle in 1 mL Tripure® reagent (Roche Diagnostics GmbH, Mannheim, Germany) and the total RNA was extracted according to the manufacturer's protocol. To remove any contaminating genomic DNA, the total RNA extracts were treated with DNase I (*DNA-free*^{TM}; Ambion Inc., Austin, UK). Then, the RNA content was quantified using a Nanodrop® spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The total RNA extracts were stored at −20°C until further use.

First-strand cDNA synthesis

Total RNA (400 ng) was mixed with 100 pmol Oligo(dT)$_{15}$ Primer (Promega, Madison, WI, USA) and RNAse free water to a total volume of 13.5 μL, incubated for 5 min at 65°C and immediately cooled on ice for 1 min. Then, 4 μL Transcriptor reverse transcriptase (RT) reaction buffer (Roche Diagnostics GmbH), 2 μL dNTP-mix (10 mM each) and 0.5 μL Transcriptor RT (10 units) (Roche Diagnostics GmbH) were added. The reaction mixture was incubated for 30 min at 55°C, followed by 5 min at 85°C and finally chilled on ice.

Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (PCR) was performed in a 25 μL PCR-reaction mixture that contained 1 μL of the primary cDNA, 12.5 μL iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and an optimalized concentration of a primer pair for one of the immunopeptide genes attacin (300 nM), defensin (700 nM) or cecropin (300 nM). The following primer pairs were used: attacinFW: 5'-TTTTCACAGTCGCACCCATT-3' and attacinREV: 5'-AAACGCCTCCTGTCAAATCC-3'; defensinFW: 5-TAG TTTTGGCTTTTCTTACAC-3' and defensinREV: 5'-CGACT ACAGTATCCGCTCTTT-3', and cecropinFW: 5'-ATACTCG CTCTTTCAGTCAG-3' and cecropinREV: 5'-CTCTAACAG TAGCGGCAACA-3'. For the amplification of the 'house keeping' genes actin (700 nM) and tubulin (300 nM), the following primer pairs were used: actin FW: 5'-CGCTTCTGG TCGTACTACT-3' and actinREV: 5'-CCGGACATCACAA TGTTGG-3', and tubulinFW: 5'-GATGGTCAAGTGCGATC CT-3' and tubulinREV: 5'-TGAGAACTCGCCTTCTTCC-3'. Reactions were run in an iCycler iQ detection system (Bio-Rad Laboratories) and analysed with its software Version 3.1. The PCR conditions comprised an initial 10-min polymerase activation at 95°C followed by 35 cycles, each consisting of a denaturation step at 95°C for 15 s and an annealing/elongation step at 60°C for 60 s. Three 'technical' replicates were performed for each sample and threshold cycles (Ct) were recorded and used to calculate gene expression levels.

Statistical analysis

All statistical analyses were carried out in STATA Version 9.2 (StataCorp, Inc., College Station, TX, U.S.A.). A linear regression was used to analyse loss of weight in individual flies. The weight difference was used as the response and fly groups (starved and non-starved teneral and adult flies) as the discrete explanatory variable. The effects of starvation on expression levels of attacin, defensin and cecropin in teneral and adult flies were analysed separately using a robust linear model. The response variables were the logarithm of the normalized number of cycles needed for the immunopeptides specific cDNA to reach the threshold in real-time PCR. The normalization used was a modification of that proposed by Vandesompele *et al*. (2002):

$$response = \ln\left[(PCR_{pep})^{Ct_{pep}}\right] - \frac{\ln\left[(PCR_{act})^{Ct_{act}}\right] + \ln\left[(PCR_{tub})^{Ct_{tub}}\right]}{2}$$

with:

- $PCR_{pep/act/tub}$ = PCR efficiency of immunopeptides, actin and tubulin, respectively;
- Ct_{pep} = number of cycles required for immunopeptide genes to reach the threshold in each sample repetition, and
- $Ct_{act/tub}$ = average number of cycles (from three repetitions) required for actin and tubulin genes, respectively, to reach the threshold in each sample.

Clustering effects resulting from repeated measures of the same samples were taken into account. Discrete explanatory variables consisted of the different experimental groups created in each experiment. Differences were considered significant when $P < 0.05$.

Results

Fat content and nutritional stress

Non-starved 20-day-old flies (AD2) had an approximately three-fold higher fat content compared with freshly emerged flies (TF0) (6.9 ± 0.2 mg vs. 2.5 ± 0.2 mg). After a period of starvation of 4 or 7 days in teneral and 20-day-old flies, respectively, a three-fold decrease in fat content was observed ($P < 0.0001$). Starving 20-day-old flies for 7 days (AD7) reduced their fat content to a level similar to that in non-starved teneral flies (TF0) (Fig. 1).

Immune gene expression level and nutritional stress

Uninduced baseline levels of immune gene expression in non-starved teneral and 20-day-old flies were similar except for that of defensins, where expression was 10-fold higher in the older flies. Starving a teneral tsetse fly for 4 days significantly reduced its baseline levels of expression of attacin and cecropin (compared with non-starved teneral flies), whereas the observed decrease in defensin expression was not statistically significant ($P = 0.07$) (Fig. 2A). In 20-day-old flies, starvation did not significantly compromise baseline expression levels of these three immune genes (Fig. 2B).

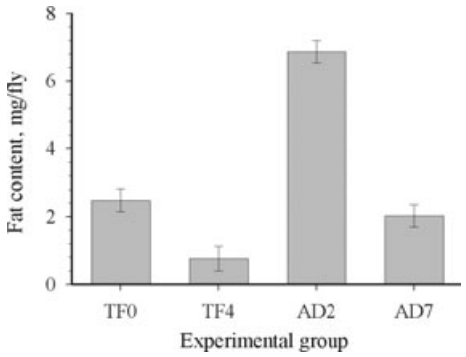

Fig. 1. Fat content (± 95% confidence intervals; $n = 24$ per group) of male teneral and 20-day-old *Glossina morsitans morsitans* after a period of starvation. The fat content was significantly reduced in teneral flies starved for 4 days (TF4) and 20-day-old flies starved for 7 days (AD7) ($P < 0.0001$) compared with non-starved teneral (TF0) and 20-day-old (AD2) flies.

Immune gene response to bacterial/trypanosomal challenge and nutritional stress

Injection of bacteria (*E. coli*) induced high levels of expression of the genes in all experimental groups. No mortality of flies was observed after the injection. In the teneral flies (Fig. 3A), the bacteria-induced increases in immune gene expression were > 100-fold for the attacin and cecropin genes and around 40-fold for the defensin gene greater than those in control flies. No significant differences were observed between the non-starved and starved flies challenged with bacteria. In the 20-day-old flies (Fig. 3B), the bacteria-induced increase in immune gene expression was around 400-fold for attacin and defensin and 1000-fold for cecropin greater than in control flies. Again, no significant difference in this immune response was observed between starved and non-starved flies.

For the trypanosomal challenge, flies were given a bloodmeal containing *T. congolense* or *T. b. brucei* bloodstream forms. Immune gene expression levels were monitored for the first 5 days following the parasite uptake. Five days after ingestion of the bloodmeal containing *T. congolense* or *T. b. brucei*, no increased expression of attacin, defensin or cecropin genes were observed in teneral tsetse flies compared with those observed on day 1 (Figs 4A, 5A). By contrast, in both non-starved and starved teneral flies challenged with *T. congolense* (Fig. 4A), gene expression levels of these immunopeptides (except for defensin) on day 5 were significantly lower than on day 1.

Non-starved 20-day-old tsetse challenged with *T. congolense* or *T. b. brucei* showed increased levels of expression of attacin, defensin and cecropin 5 days after the infective bloodmeal compared with day 1 (Figs 4B, 5B), although this increase was demonstrated as statistically significant only for the *T. b. brucei*-infected group. No significant changes in immune gene expression levels were observed in the starved 20-day-old tsetse fly group after a bloodmeal containing *T. congolense* or *T. b. brucei*.

Discussion

Trypanosome transmission is compromised by a natural tsetse fly refractoriness to trypanosome infection because only a small subset of flies allow the development of a mature infection in the hypopharynx (*T. congolense*) or salivary glands (*T. b. brucei*). One of the defence mechanisms implicated in this refractoriness is the tsetse fly's immune response. An important part of this immune response is based upon the expression of several AMPs that are mainly produced systemically by the fat body (Hao *et al.*, 2001; Boulanger *et al.*, 2002). In addition, it was shown that the nutritional status of the tsetse fly at the time of the infective bloodmeal affects its vectorial ability for both *T. congolense* and *T. b. brucei*. Indeed, an extreme period of starvation (4 days for freshly emerged flies, 7 days for older flies) lowers the developmental barrier for trypanosome infection, especially at the midgut

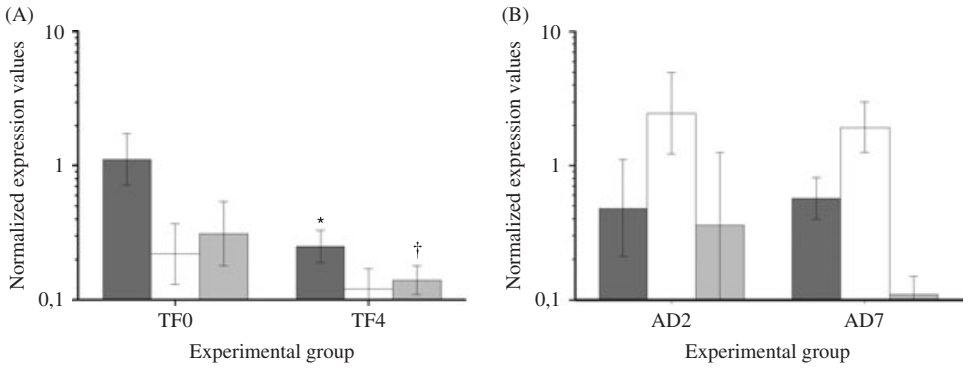

Fig. 2. Normalized expression levels (± 95% confidence intervals; $n = 12$ per group) of attacin (dark grey bars), defensin (white bars) and cecropin (light grey bars) in male teneral and 20-day-old *Glossina morsitans morsitans* after starvation. Expression levels (mRNA) of attacin, defensin and cecropin were measured in (A) non-starved (TF0) and starved (TF4) teneral and (B) non-starved (AD2) and starved (AD7) 20-day-old flies using quantitative real-time polymerase chain reaction. Immunopeptide gene expression values were normalized against the expression levels of the housekeeping genes actin and tubulin. The expression levels of attacin and cecropin were significantly reduced in teneral flies starved for 4 days (TF4) compared with non-starved teneral flies (TF0) (*$P < 0.0001$, †$P < 0.02$).

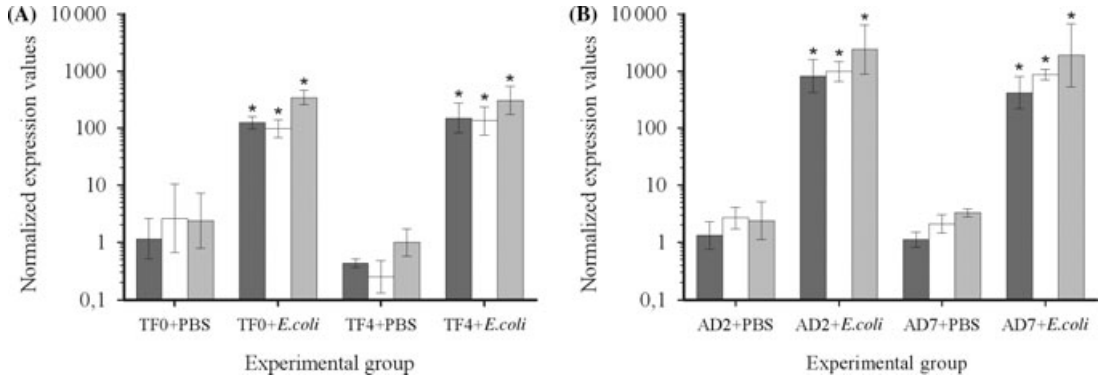

Fig. 3. Normalized expression levels (± 95% confidence intervals; $n = 4$ per group) of attacin (dark grey bars), defensin (white bars) and cecropin (light grey bars) in (A) non-starved (TF0) and starved (TF4) teneral and (B) non-starved (AD2) and starved (AD7) 20-day-old male *Glossina morsitans morsitans* after bacterial (*Escherichia coli*) challenge. The immunopeptide gene expression values were normalized against the expression levels of the housekeeping genes actin and tubulin. Expression levels of the three antimicrobial peptides were highly upregulated in the *E. coli*-stimulated flies (*$P < 0.0001$). PBS, phosphate buffered saline.

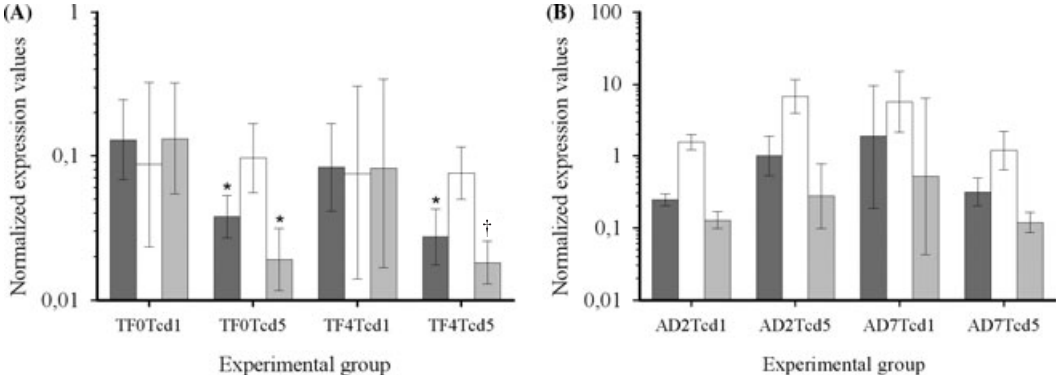

Fig. 4. Normalized gene expression levels (± 95% confidence intervals; $n = 5$ per group) of attacin (dark grey bars), defensin (white bars) and cecropin (light grey bars) in (A) non-starved (TF0) and starved (TF4) teneral and (B) non-starved (AD2) and starved (AD7) 20-day-old male *Glossina morsitans morsitans* on day 1 (d1) or day 5 (d5) after uptake of a bloodmeal containing *Trypanosoma congolense* (Tc) bloodstream forms. Immunopeptide gene expression values were normalized against the expression levels of the housekeeping genes actin and tubulin. The expression levels of attacin and cecropin were significantly reduced in teneral flies starved for 4 days (TF4) compared with non-starved teneral flies (TF0) (*$P < 0.0001$, †$P < 0.03$).

level of the fly (Kubi *et al*., 2006). Hence, in this study, the effect of starvation (= nutritional stress) was evaluated on the uninduced baseline levels of gene expression of the AMPs attacin, defensin and cecropin in the tsetse fly and their induced levels of expression in response to bacterial (*E. coli*) or trypanosomal challenge. The injection of bacteria causes a direct and strong stimulation of the tsetse fat body immune response (Hao *et al*., 2001; Boulanger *et al*., 2002) and is an appropriate assay to evaluate whether the responsiveness of the fly's major immune responsive organ is affected by nutritional deprivation. Ingestion of trypanosome parasites in the tsetse midgut is also reported to indirectly induce a differential fat body response, but the exact nature of this trypanosome-related stimulus is not yet clearly understood (Lehane *et al*., 2008). In addition, local production of specific AMPs in the tsetse midgut in response to trypanosome parasites cannot be excluded as the gut of a range of insects was found to be a site of AMP synthesis (Boulanger *et al*., 2006). However, the AMP gene expression analysis performed in this study did not allow us to make this distinction between local and systemic immune response because it was based on RNA that was extracted from the whole fly abdomen containing both the fat body and the tsetse midgut.

Both freshly emerged and 20-day-old *G. m. morsitans* flies showed significant baseline gene expression of all three AMPs, with a 10-fold increase in defensin expression observed in the 20-day-old flies. However, starving freshly emerged flies for 4 days to a fat reserve level of < 1 mg/fly significantly reduced this level of immune gene expression. This suggests that high nutritional stress in these young flies results in a significant reduction in uninduced baseline immune gene expression (i.e. innate expression before acquisition of trypanosomes), which, in turn, may contribute to the increased susceptibility of these flies to trypanosome infection. Indeed, a correlation between this baseline immune gene level and the fly's susceptibility to trypanosome infection was recently suggested by Nayduch

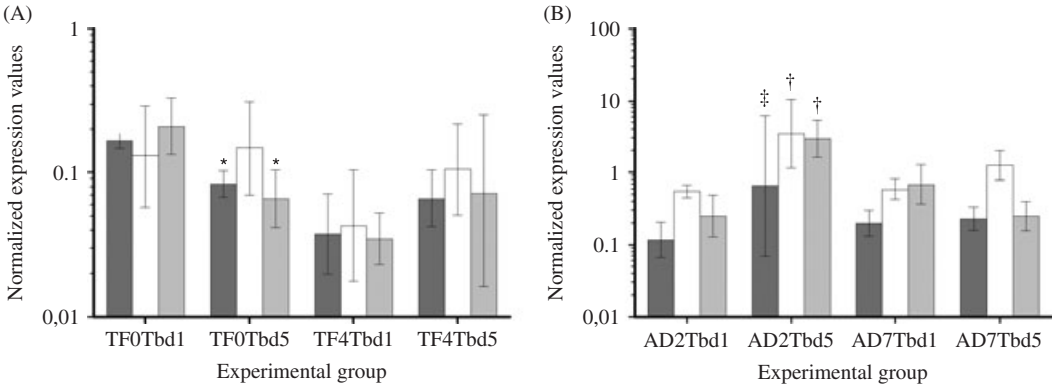

Fig. 5. Normalized gene expression levels (± 95% confidence intervals; $n = 5$ per group) of attacin (dark grey bars), defensin (white bars) and cecropin (light grey bars) in (A) non-starved (TF0) and starved (TF4) teneral flies and (B) non-starved (AD2) and starved (AD7) 20-day-old male *Glossina morsitans morsitans* on day 1 (d1) or day 5 (d5) after uptake of a bloodmeal containing *Trypanosoma brucei brucei* (Tb) bloodstream forms. Immunopeptide gene expression values were normalized against the expression levels of the housekeeping genes actin and tubulin. The expression levels of the immunopeptide genes were significantly upregulated in non-starved adult flies after a bloodmeal with *T. brucei* bloodstream forms (*$P < 0.0001$, †$P < 0.03$, ‡$P = 0.06$).

& Aksoy (2007), who showed that the uninduced baseline level of systemic attacin was significantly higher in freshly emerged flies of trypanosome-refractory tsetse species than in susceptible species. In 20-day-old flies, the effects of nutritional stress by starvation were less pronounced (i.e. a three-fold reduction in fat level, which is comparable with that in non-starved teneral flies), which allowed the flies to maintain baseline levels of immunopeptide expression similar to those in non-starved flies.

A bacterial challenge by live *E. coli* injection resulted in high increases in expression of attacin, defensin and cecropin in all experimental fly groups, thus confirming the strong immunogenic nature of an *E. coli* injection to tsetse flies (Hao *et al*., 2001; Boulanger *et al*., 2002; Lehane *et al*., 2004). Flies aged 20 days showed a much higher degree of responsiveness to the bacterial challenge than freshly emerged flies, with a 400-fold induction for attacin and defensin and a 1000-fold induction for cecropin in comparison with uninduced baseline levels. These data clearly show that the fat body, as the major immune response organ, is operational in freshly emerged, unfed flies, but has not yet attained its full capacity to respond to pathogens. Moreover, as no differences were found between the starved and non-starved groups, it is clear that this immune responsiveness to the bacterial challenge is not affected by the nutritional status of the flies. This indicates that although the maintenance of a powerful immune defence system represents a high cost in energy for the tsetse fly (Schmid-Hempel, 2005), keeping a properly functioning and alert immune defence system is of vital importance to the fly, even when its energy reserve is seriously depleted. The uptake of approximately 5×10^6 bloodstream trypanosomes (*T. congolense* or *T. b. brucei*) by blood-feeding did not affect expression levels of attacin, defensin and cecropin in the teneral fly groups at 5 days after the infective bloodmeal, which confirms previous observations by Hao *et al*. (2001) that the immune system response to ingested bloodstream trypanosomes in young tsetse flies is low. In the adult fly group, gene expression of attacin, defensin and cecropin in response to trypanosome infection (especially for *T. b. brucei*) increased significantly only in non-starved flies; this may represent a contributing factor to the high refractoriness for trypanosome infection observed in these flies (Kubi *et al*., 2006).

In conclusion, this study reports that high nutritional stress decreases baseline immunopeptide gene expression in newly hatched unfed tsetse flies and decreases the immune responsiveness of older flies to trypanosome challenge. This decreased immune gene expression as a result of starvation may contribute to the increased susceptibility of nutritionally stressed tsetse flies to developing a trypanosome infection. It is clear that the present study is based only on a limited experimental read-out of three well-characterized immune-responsive genes in tsetse (attacin, defensin and cecropin), regulated by the IMD pathway, which are assumed to represent a barometer for the functioning of the fat body-regulated immune response. It would be worthwhile expanding this starvation study to the expression of other fat body-regulated genes that are differentially expressed in trypanosome-challenged tsetse flies.

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