

equation are between -2.9% and $+2.2\%$, while for the range $0.02\text{ m} < H < 0.06\text{ m}$, according to Rehbock equation, they are between -2.8% and $+2.3\%$ (Figure 6).

Using the artificial finger, the discharge can be defined within error limits of -2.9% and $+2.3\%$ using discharge formulas from the international standards suitable for the range of measurement, as described above. Compared to reports in the literature, this is a new finding, since the literature recommends an equation for ventilated nappe calculation (without indicating error limits), which is not included in the international standards.

Since these functions do not depend on Re and We , unlike those in the literature, the influence of these numbers may be lowered from $H \geq 0.03\text{ m}$

- to $H \geq 0.0171\text{ m}$ for increase in discharge and
- to $H \geq 0.01\text{ m}$ for decrease in discharge.

For non-ventilated nappe, in case of $H \geq 0.004\text{ m}$ (Figures 7 and 8)

- Without the artificial finger, error ranges between -5.7% and $+3.8\%$ in variant A and between -2.9% and $+2.6\%$ in variant B.
- With the artificial finger, the error limits are between -2.8% and $+2.3\%$ in variant A and between -2.2% and $+3.7\%$ in variant B.

Use of artificial finger for non-ventilated nappe improves the measurement of discharge in variant A to the highest degree, where errors are between -2.8% and $+2.3\%$.

While the literature recommends two equations for non-ventilated nappe, in this study we use only one equation with the given error limits.

Thus, the use of artificial finger enabled the discharge hydrograph measurement of free overflow using the full-width, thin-plate weir.

Further tests should specify the location of the artificial finger in a full-width thin-plate weir with free flow (non-submerged) ventilated discharge as the function of discharge height P and width B .

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Modulation of midgut peritrophins' expression during *Plasmodium* infection in *Anopheles stephensi* (Diptera: Culicidae)

V. Venkat Rao¹, Surendra Kumar Kolli¹, Shruti Bargava¹ and R. K. Chaitanya^{2,*}

¹Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

²Centre for Animal Sciences, Central University of Punjab, Bathinda 151 001, India

The peritrophic matrix (PM) serves as a barrier to pathogens in many disease vectors including mosquitoes. The *Plasmodium* ookinete has to cross the PM barrier for its successful establishment in the mosquito mid-gut and subsequent transmission. It is conceived that alterations to PM may lead to a block in infection.

*For correspondence. (e-mail: chaitanyark@gmail.com)

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Peritrophins which are the major constituents of PM are yet to be elucidated at molecular level. The present study demonstrates *Anopheles stephensi* midgut peritrophins' expression during *Plasmodium berghei* infection. Eight peritrophin genes (*Per 10*, *Per 16*, *Per 22*, *Per 25*, *Per 26*, *Per 28*, *Per 30* & *Per 43*) of *A. stephensi* were identified from vectorbase, isolated from the adult midgut, and expression pattern monitored in real-time, in normal and infected blood meal conditions. Temporal expression of peritrophins in the midgut was monitored every 6 h till 24 h post blood meal. Results showed that the *Per 10*, *Per 16*, *Per 22*, *Per 25* and *Per 26* expression was significantly downregulated during *Plasmodium* infection whereas *Per 30* and *Per 43* expression was markedly up-regulated. The *Per 28* expression was low initially but elevated later. This data clearly indicates that peritrophins are differentially modulated in infected midgut. The significance of differential expression of peritrophins' in parasite transmission is discussed further.

Keywords: *Anopheles stephensi*, malaria, peritrophins, *Plasmodium berghei*, transmission.

IN most insects, the midgut epithelium is striated with an acellular, semi-permeable structure known as peritrophic matrix (PM). The PM acts as a molecular sieve which mediates circulation of molecules to and from the midgut lumen and prevents clogging of the microvilli by luminal contents, thereby acting as a barrier and protecting hosts from xenobiotics and toxins^{1,2}. Principal components of PM are chitin fibrils and glycoproteins. PM is secreted either by the midgut epithelium (type I) or the cardia (type II). Type I PM is formed by delamination from the surface of the midgut in response to feeding and/or the type of meal ingested (primarily in lepidopteran larvae and dipteran adults). Type II PM is constitutively synthesized independent of food ingestion (primarily in dipteran larvae)³. PM proteins are grouped into four classes based on their differential extraction using solubilizing agents. Class I proteins can be removed with physiological buffers and therefore represent loosely associated proteins. Class II proteins are extractable with mild detergents, such as sodium dodecyl sulphate, which disrupt weak ionic interactions. Class III proteins are integral to PM and released only with strong denaturants such as urea, guanidine hydrochloride, etc. Class IV proteins are covalently linked to chitin or other proteins and cannot be removed^{2,4}. Class III proteins, collectively known as peritrophins, are the most extensively studied proteins. Every identified peritrophin so far has registers of 6, 8, or 10 cysteine residues referred to as peritrophin domains A, B or C respectively that are involved in chitin interactions. Mucin-like peritrophins characterized by proline-, serine-, and threonine-rich domains were also extracted from PM. These proteins possess water retaining capability and hence protect the tissue from dehydration and provide

lubrication for the food passage³. Peritrophins have been identified in several arthropods such as tobacco hornworm, *Manduca sexta*⁵, Bertha armyworm, *Mamestra configurata*⁶, Beet webworm, *Loxostege sticticalis*⁷, Cat flea, *Ctenocephalides felis*⁸, myiasis fly, *Chrysomya bezeliana*⁹, Australian sheep blowfly, *Lucilia cuprina*¹⁰, Tsetse fly¹¹, *Anopheles gambiae*¹², *Anopheles albimanus*¹³ and *Aedes aegypti*¹⁴.

The PM of hematophagous insects, particularly mosquitoes, has been demonstrated to have an added role of limiting *Plasmodium* establishment in the mosquito midgut. *Plasmodium*-derived chitinases disrupt the chitin framework of PM and ensures successful parasite traversal^{15,16} in the *Anopheles* species. However, mosquito-encoded trypsin is required for activating the chitinase^{17,18}. Although we now know that malaria parasite secretes a chitinase to facilitate the penetration of PM, the specific role of peritrophins during *Plasmodium* infection is not elucidated so far. In the present study, we report modulated expression of peritrophin genes during *Plasmodium berghei* infection of *Anopheles stephensi*.

A. stephensi adults were reared in a closed chamber maintained at 27°C under 70% humid conditions with a 12-h light–dark cycle on 10% sucrose solution. *P. berghei* ANKA strain was maintained in 3–4-week-old female BALB/c mice or as frozen stocks. The mice with parasitemia between 4% and 8% as checked by Giemsa staining were used to infect mosquitoes. All the animal experiments were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments in Animals (CPCSEA, India) as well as approval by the Institutional Animal Ethics Committee (School of Life Sciences, University of Hyderabad, India).

Female anopheles mosquitoes (2–4 days old) infected with *P. berghei* were fed on anesthetized infected BALB/c mice for 15 min. Control and infected mosquitoes were kept in a chamber maintained at 21°C and 70% humidity. *P. berghei* midgut infection was confirmed by monitoring chitinase mRNA expression using quantitative real-time PCR (qRT-PCR). The primers are listed in Table 1.

Mosquitoes ($n = 40$) from normal and infected blood fed groups were rinsed with 70% (v/v) ethanol and the midguts were dissected under microscope. The midguts obtained from different time points (0, 6, 12, 18 and 24 h) were collected in 200 μ l of TRI[®] reagent (Sigma-Aldrich, USA), snap frozen in liquid nitrogen and stored at –80°C till further use.

Peritrophin sequences of *A. stephensi* were obtained from VectorBase (ASTEI06523, ASTEI11436, ASTE001076, ASTEI09413, ASTEI00139, ASTEI06725, ASTEI09414, ASTEI09414). Clustal Omega tool was applied for sequence alignment. Signal peptide prediction was performed using SignalP 4.0 version. NetNGlyc and NetOGlycsoftwares were used for prediction of potential

Table 1. List of primers used for cloning and real-time analysis of *A. stephensi* peritrophin genes

Gene	Forward	Reverse
<i>Per 10</i>	5'-ATGAAGGAGTTGGTGCATTG-3'	5'CTATCCAAACTTATCGGATC-3'
<i>Per 16</i>	5'-ATGAAAGCTTCCGGTGCCTTG-3'	5'-TTATTCCTCACACCCTGCTAGC-3'
<i>Per 22</i>	5'-ATGGATTATCAGTCTTACT-3'	5'-GCCCATCCAAGATTGGATTTCG-3'
<i>Per 25</i>	5'-ATGGCACGGCACGCCTTGAC-3'	5'-CTATTTCAACAGACTCGAGCAG-3'
<i>Per 26</i>	5'-ATGGCAGGCCAACTTTCAACGG-3'	5'-CTAAACAGCTTTAGTGCAGTAAG-3'
<i>Per 28</i>	5'-GTTATTGCCAGATGCCACTGCAT-3'	5'-TCACTGCCTTGTGCAGTATCGCA-3'
<i>Per 30</i>	5'-ATGAAGACTGTGTGCAGTGTCTTG-3'	5'-TTAATTGTGTGAAAAGTGCATG-3'
<i>Per 43</i>	5'-ATGATGCCAGGTTGGTCCATAATAC-3'	5'-CTAGTATCCATCATTCTCACACTC-3'
RT <i>Per 10</i>	5'-TACGTTTCGCTGTAGCCGTT-3'	5'-TCAATGTTCCAGTGCAAAACC-3'
RT <i>Per 16</i>	5'-GATGGAAGAAATGTGCGA-3'	5'-TGTAGTACTTGGTGCAGTCCGT-3'
RT <i>Per 22</i>	5'-CGAGAGCAACTGTTTCAAGT-3'	5'-GCCCAAAGATTCTTCTCTCT-3'
RT <i>Per 25</i>	5'-GTACCTTCCCACATCCAACC-3'	5'-GTTGTAGTTGATGCGTCCGT-3'
RT <i>Per 26</i>	5'-GCGATAGCTATGGAGATCCG-3'	5'-CAGATCGCACAGATCGAAGT-3'
RT <i>Per 28</i>	5'-CGATCCCAACAATTGCTACA-3'	5'-GATCCAACAGGACACGTCAC-3'
RT <i>Per 30</i>	5'-TGTGTGCAGTGTCTTGTGT-3'	5'-GCAGTGCAGTGAACAGAAT-3'
RT <i>Per 43</i>	5'-CCAAGTCCGATCATTACG-3'	5'-ACTCGACCGAATGTTCCACT-3'
<i>As rS7</i>	5'-ATGAAGCTCGGATCTGAAGCC-3'	5'-CTTCTTCTCCAATTACGCA-3'

glycosylation sites. ExPASy-Compute pI/Mw tool was used for prediction of putative molecular weight and isoelectric point of the peritrophins.

Prior to real-time analysis, full-length midgut peritrophin genes were amplified and cloned into pTZ57R/T vector, sequenced and confirmed by BLAST analysis. For qRT analysis, control and infected midgut samples were collected at different time points (0, 6, 12, 18 and 24 h) in ice-cold TRI[®] reagent (Sigma-Aldrich) and total RNA was isolated. All the RNA samples were treated with DNase I prior to first strand cDNA synthesis to eliminate any possible DNA contamination. First-strand cDNA synthesis was carried out using Superscript III[®] following manufacturer's protocol. The list of quantitative RT-PCR primers is given in Table 1. Gene expression was assessed by SYBR green qRT-PCR master mix (Applied Biosystems) using ABI-7500 fast real-time PCR system (Applied Biosystems). A 40-cycle PCR was carried out in triplicates with 20 µl reaction volume containing the following components: 1 µl of cDNA template, 1 µl of forward and reverse primers each (various dilutions), and 10 µl of 2X master mix and 7 µl of nuclease-free water. The amplification efficiency was 95–99% with slope of the curve ranging between –3.0 and –3.3. During each cycle of PCR, fluorescence accumulation resulting from DNA amplification was analysed and converted into cycle threshold (C_t) by the sequence detection system software (Applied Biosystems). Relative quantification results were normalized with *A. stephensi* ribosomal protein S7 as endogenous internal control. All the results were represented as relative fold change to the reference values obtained for control and normalized to that of endogenous control gene (S7) C_t values using the $2^{-\Delta\Delta C_t}$ method.

Data are expressed as mean \pm SEM of three independent experiments ($n = 3$). Two-way ANOVA was used to

test for significant differences in the expression patterns of genes in midguts under normal and infected conditions (Systat Software Inc., USA). A probability of $P < 0.05$ was considered statistically significant.

ClustalW alignment showed significant homology between *A. gambiae* and *A. stephensi* peritrophins (Figure 1). The *A. stephensi* peritrophins obtained from Vector-Base were designated as *Per 10*, *Per 16*, *Per 22*, *Per 25*, *Per 26*, *Per 28*, *Per 30* and *Per 43* based on their calculated molecular weights respectively. It is evident that peritrophins possess low molecular weight ranging from 22 kDa to 42 kDa. However, the presence of potential glycosylation sites is expected to add up to the molecular weight of secreted protein. Potential N-linked glycosylation sites were found in all peritrophin sequences excluding *Per 10* and *Per 16*. Multiple O-linked glycosylation sites were also located in the peritrophin sequences with an exception of *Per 10* and *Per 26*. Further, all peritrophins display predicted signal peptides suggesting that these proteins are secreted into the midgut lumen. Chitin binding domains that are characteristic of peritrophins were present in all the mature proteins analysed, although the number of domains as well as cysteine residues harboured in them varied. Further, the theoretical pI of peritrophins was in the range of 4–7. It is noteworthy that none of the peritrophins contained heme-binding domains in them (Table 2).

Midgut peritrophin expression was monitored at every 6 h interval post blood meal till 24 h. The results depict that the peritrophin genes were temporally regulated. *Per 28* expression was high initially at a very early stage which markedly declined by 6 h post blood meal and remained unaltered thereafter till 24 h. Conversely, *Per 16*, *Per 22*, *Per 25*, *Per 26* and *Per 30* expression levels were unaltered till 6 h. However, the expression was upregulated steeply from 6 to 12 h post blood meal following

Table 2. *In silico* analysis of *A. stephensi* peritrophins

Vector base ID	Putative/ deduced mol. wt. kDa	Signal peptide seq	CBDs	Peritrophin A/B/C/ based on 6/8/10 cysteines	Heme binding domains	Glycosylation (N or O)	PI
ASTE106523	10	19–20	1	5	No	0/0	4.43
ASTE111436	16	23–24	2	4, 5	No	0/2	4.52
ASTE001076	22	23–24	2	6, 5	No	1/2	4.68
ASTE109413	25	23–24	3	6, 6, 6	No	2/15	5.86
ASTE100139	26	26–27	1	11	No	2/0	6.40
ASTE106725	28	18–19	2	4, 3	No	1/21	6.08
ASTE109414	30	20–21	2	5, 5	No	2/10	5.66
ASTE110276	42	21–22	4	5, 5, 5, 6	No	1/2	7.11
ASTE110274	99	No	2	6, 6	No	5/46	8.12

which a sharp decline was observed from 12 to 18 h. Subsequently, the expression levels only marginally increased from 18 h onwards. *Per 10* expression gradually increased and peaked towards the end of the blood meal regimen from 18 to 24 h. However, *Per 43* expression remained unaltered during the monitored time period (Figure 2).

Most of the midgut peritrophins of the mosquitoes that were fed with parasite-infected blood were down-regulated. *Per 10*, *Per 16*, *Per 22*, *Per 25* and *Per 26* expression remained significantly low when compared with blood-fed control. Although *Per 28* expression was low initially, it elevated rapidly from 18 to 24 h. On the other hand, *Per 30* and *Per 43* expression markedly up-regulated compared to the control (Figure 2).

In *A. gambiae*, more than 200 proteins have been reported in the proteome of peritrophic matrix, of which twelve are peritrophins¹⁹. Based on the sequence similarity with *A. gambiae* peritrophins, presence of secretory signal peptide, chitin-binding domain (s) and glycosylation sites, eight putative peritrophins were identified in *A. stephensi*. The absence of serine/proline/threonine-rich domains and the heme-binding domains further confirm that these proteins are different from the mucin-like peritrophins³, which are also reported under Class III peritrophic matrix proteins^{4,14}. The presence of a varied number of chitin-binding domains in *A. stephensi* peritrophins might arise from a common ancestor protein harbouring a single chitin-binding domain (CBD) as revealed earlier²⁰. CBD domains are represented by the conservation of cysteine residues that conform to the general pattern of CX13-20-CX5-6CX9-19CX10-14CX4-14C (where X can be any amino acid)^{3,4}. The duplication and transposition of chitin-binding domain was predicted to contribute to the functional diversification of chitin-binding proteins identified in both parasite-secreted chitinases and host-secreted PM proteins^{20,21}. The disulphide bonds formed by cysteine residues in CBD ensure strong resistance of PM proteins against proteolytic digestion by various digestive proteases secreted in high concentrations in the mosquito gut lumen after a blood meal. In addition, tryp-

sin and chymotrypsin cleavage sites are generally located within the CBD and are thus inaccessible to proteases^{3,22}. However, mosquito midgut membrane-bound proteases such as aminopeptidase and carboxypeptidase are postulated to be ideal candidates for blocking transmission. Therefore, it would be intriguing to elucidate the interaction of peritrophins with these digestive enzymes during normal and infected blood^{3,19}.

The absence of a PM in adult male mosquitoes implies that the female mosquito PM has a role during blood feeding. Further, the blood meal-induced PM is highly dynamic and is capable of manipulating its thickness and porosity to varying degrees¹. This varying degree of thickness of PM could be due to differential peritrophin expression observed. In the present study, it was observed that most of the peritrophin genes were upregulated by 24 h following blood meal which corroborates with maximum thickness of peritrophic matrix observed at the same time¹⁹.

Earlier, Dana *et al.*²³ demonstrated that the midgut transcripts including peritrophins were present as early as 30 min post-blood meal and further categorized the time-dependent differential expression of peritrophins in *A. gambiae* into early-, mid- and late-phase genes. Further, a previous report showed the absence of secretory vesicles present in the apical brush border of midgut epithelial cells of adult female mosquito in as early as 1 h post blood meal²⁴. Based on these reports and the temporal expression pattern of peritrophins obtained in the current study, it can be presumed that the early peritrophin genes may be transcribed in response to the blood meal and their corresponding proteins are used immediately in the formation of the peritrophic matrix whereas the middle and late peritrophin gene products may be packaged into vesicles in preparation for a subsequent blood meal. Temporal regulation of secretory vesicular appearance process where PM precursors are accumulated was also demonstrated in the midgut of *A. darlingi*²⁵, *A. albimanus*¹³ and *A. stephensi*²⁶. In contrast, in *A. aegypti*, the vesicles are formed *de novo* after blood feeding¹⁹. The early-, mid- and late-stage peritrophin expression in the *A. stephensi* midgut observed in our study corroborates

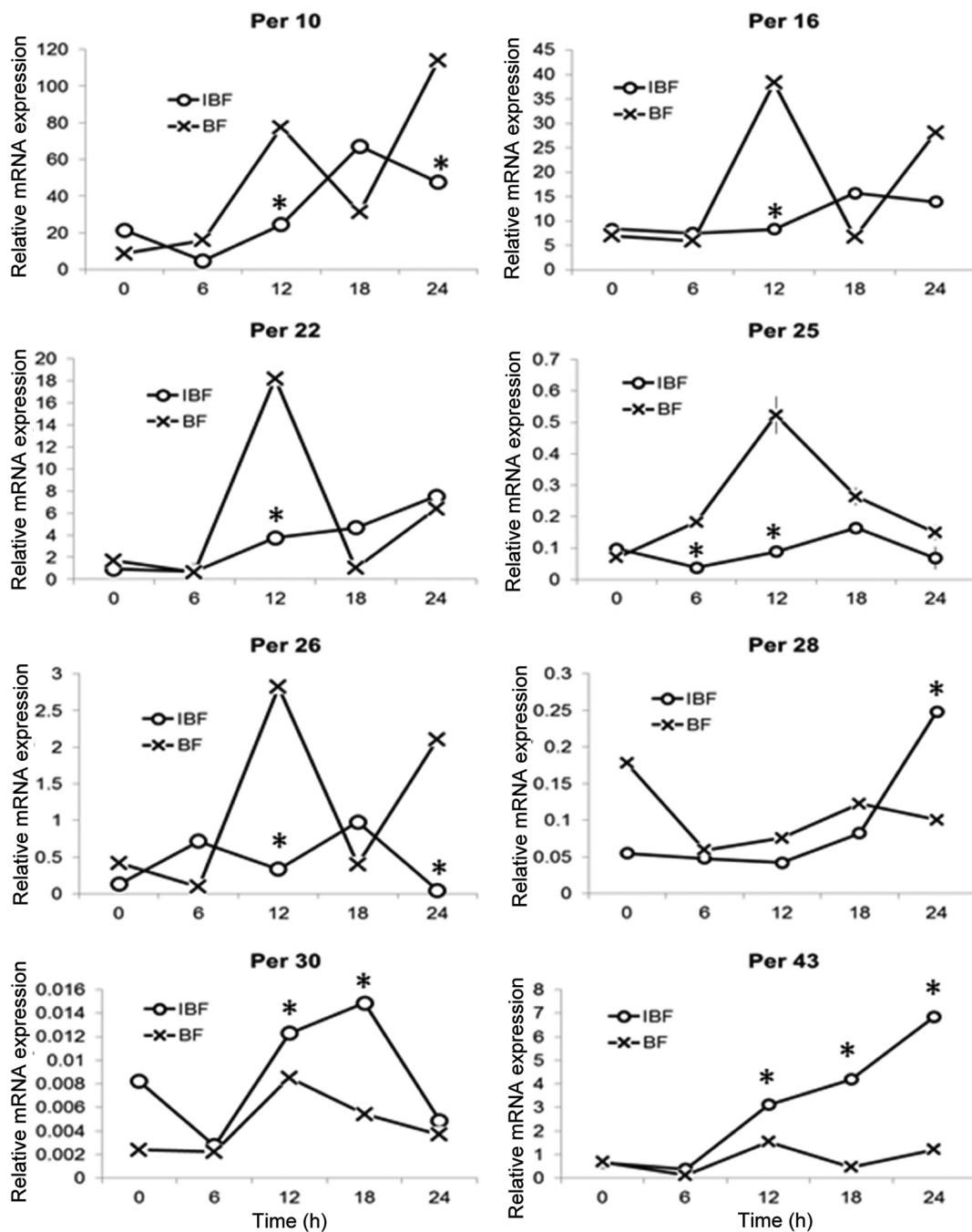


Figure 2. Expression of peritrophin genes in *A. stephensi* mosquitoes during *P. berghei* infection relative to control. *Denotes significance between control and infected samples ($P \leq 0.05$) at a given time point.

with previous studies. However, whether the induction of PM components including the peritrophins post blood meal is only direct (presence of blood in the midgut) or indirect (via endocrine pathways) remains to be investigated. Circumstantial evidences point out the role of morphogenetic hormones in the regulation of the PM formation and structure. 20-Hydroxyecdysone increased PM production in *Calliphora erythrocephala* whereas juvenile hormone suppressed it²⁷.

The role of peritrophins and their expression during mosquito hematophagy is currently being explored. However, there is no information of their participation, if any, during infection. To traverse the gut epithelium, the ookinete has to breach through PM and therefore might constitute a potential barrier for *Plasmodium* invasion. Whether the PM is a physical barrier for the parasite has been examined by artificial manipulation of the thickness of PM which resulted in decreased *Plasmodium* infectivity²⁸. In a

recent study, it was shown that PM influences parasitic microbial infection outcome in the Tsetse flies (*Glossina* sp.)¹¹. Our data provides the first definitive evidence of variations in peritrophin expression during *Plasmodium* infection. Most peritrophin genes were down regulated upon infection, which could result in decreased peritrophin synthesis and secretion and altered peritrophic matrix configuration. This study indicates a direct interaction between the parasite and midgut peritrophins. Hence, chemical or immunological interference would not only specify the parasite–peritrophin interaction but also may help in identifying potential molecular target (specific) peritrophin which could block *Plasmodium* transmission within the *Anopheles* mosquito. Further studies will be focussed in this direction.

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