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RESEARCH ARTICLE

Malaria *Plasmodium* agent induces alteration in the head proteome of their *Anopheles* mosquito host

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Despite increasing evidence of behavioural manipulation of their vectors by pathogens, the underlying mechanisms causing infected vectors to act in ways that benefit pathogen transmission remain enigmatic in most cases. Here, 2-D DIGE coupled with MS were employed to analyse and compare the head proteome of mosquitoes (*Anopheles gambiae sensu stricto* (Giles)) infected with the malarial parasite (*Plasmodium berghei*) with that of uninfected mosquitoes. This approach detected altered levels of 12 protein spots in the head of mosquitoes infected with sporozoites. These proteins were subsequently identified using MS and functionally classified as belonging to metabolic, synaptic, molecular chaperone, signalling, and cytoskeletal groups. Our results indicate an altered energy metabolism in the head of sporozoite-infected mosquitoes. Some of the up-/down-regulated proteins identified, such as synapse-associated protein, 14-3-3 protein and calmodulin, have previously been shown to play critical roles in the CNS of both invertebrates and vertebrates. Furthermore, a heat shock response (HSP 20) and a variation of cytoarchitecture (tropomyosins) have been shown. Discovery of these proteins sheds light on potential molecular mechanisms that underlie behavioural modifications and offers new insights into the study of intimate interactions between *Plasmodium* and its *Anopheles* vector.

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1 Introduction

The continuing co-evolutionary 'arms race' between parasites and their hosts results in many fascinating adaptations [1]. Parasites evolve to optimise their transmission and/or host exploitation, whilst hosts evolve to minimise the parasite-induced fitness loss. Such a conflict systematically occurs in insect vector-pathogen systems when, for instance, vectors prefer to bite their vertebrate hosts at a frequency that is less than optimal for transmissible stages of the parasite [2, 3]. In this context, many of the most harmful pathogens have been shown to manipulate the behaviour of their vectors, such as feeding behaviour, in ways that increase the contact with the vertebrate host and hence favour pathogen transmission [4]. Several studies with different systems support the idea that parasites indeed increase the probing and feeding rate of their vectors by a variety of mechanisms, including, obscuring phagoreceptors, blocking the foregut and re-

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Abbreviations: C, control; **MI**, mature infection; **NI**, non-infective; **NO,** nitric oxide

ducing apyrase activity in salivary glands [4]. In all cases, these mechanisms seem to impair the vector's ability to fully engorge and therefore induce them to feed several times on vertebrate hosts.

In the malaria-mosquito system, evidence continues to accumulate showing that *Plasmodium* spp. manipulate the behaviour of their mosquito vectors, *Anopheles* spp. [5–11]. In this system, the altered behaviour often takes place when sporozoites have invaded the salivary glands, *i.e.* once the parasite reaches the infective stage for the vertebrate host [7, 9, 10]. For instance, malaria parasites (*e.g. Plasmodium yoelii nigeriensis, P. gallinaceum*) manipulate their mosquito vectors in two different ways and in a stage-specific manner: when they have reached the sporozoite stage and are ready to be transmitted to the vertebrate hosts, the parasite increases the biting rate of its vector [9–11]. In contrast, when at the oocyst stage and not transmissible to the vertebrate host, the parasite decreases the contact between vector and vertebrate host by decreasing the natural host-seeking behaviour of the insect [10, 11]. Since biting is risky and could lead to the insect's death, this change is beneficial for the parasite.

Our understanding of the molecular and physiological bases of insect vector behaviour is currently rather poor [12]. The CNS functions primarily to convert patterns of activity in sensory receptors into patterns of muscle activity that constitute appropriate behaviour. Thus, any changes in insect vector behaviour would be expected to have a molecular basis in its CNS. Exploring this molecular basis will undoubtedly help to elucidate how pathogens manipulate the behaviour of their insect vector. An investigation of parasite-induced effects on host CNS proteome expression may play a crucial role in this respect [13], especially in insect vector-pathogen systems [14]. Proteomics, with the ability to investigate the translation of genomic information, offers an approach to study the global changes in protein expression of the host CNS caused by parasites [15–17]. Here, we have applied such an approach to the *Anopheles gambiae-Plasmodium berghei* (murine malaria) experimental model to elucidate the molecular mechanisms in the brain that underlie behavioural modifications.

Using 2-D DIGE coupled with MS, the current study aims to identify proteins with altered levels within the CNS between three groups of mosquitoes: (i) mosquitoes fed with an infective blood meal, the parasite being in the salivary glands (sporozoite stage), *i.e.* "mature infection" (MI); (ii) mosquitoes fed with a blood meal containing the mutant parasite (2.33) that does not produce gametocytes, *i.e.* "non-infective"(NI); and (iii), mosquitoes fed with non-infectious blood meal, *i.e.* "control" (C). The NI group was used as a second control to exclude proteins that are non-specific to mature infection and hence unlikely to be linked with behavioural changes [18]. To avoid cohort effects, this experiment was repeated with a second cohort of mosquitoes 1 month later. Thus, six samples were analysed: MI_1, NI_1, C_1 from the first cohort and MI_2, NI_2, C_2 from the second cohort.

2 Materials and methods

2.1 Mosquitoes

The Yaounde strain of *Anopheles gambiae* was used for all assays reported here. This strain originated from Yaounde, Cameroon [19], and is maintained under standard laboratory rearing conditions at the IBMC, Strasbourg, France. Prior to blood feeding (day 0), mosquitoes were placed in containers at a density of 50–75 females/cup, sugar starved for 6 h before a blood meal and then allowed to feed for 30 min on an anaesthetised mouse. The mosquitoes received one of three types of blood meals: (i) Pb-GFPcon, a transgenic strain of *P. berghei* 2.34 that expresses GFP under the control of *elongation factor 1* (*eLF1*) throughout the entire parasite lifecycle [20] (MI); (ii) blood meal from a 2.33 *Plasmodium berghei* infected mouse (2.33 is a mutant strain of *P. berghei* that does not produce gametocytes (NI)); and (iii) non-infectious blood meal (C). All non-blood-fed mosquitoes were removed from the cups and not included in the analyses. Blood-fed mosquitoes were maintained at 21°C for the remainder of the assay. We tried to perform a third control category consisting of mosquitoes infected with the oocyst (non-transmissible) stage, but failed to obtain enough material.

At day 20 post-blood meal, mosquito heads were collected for further analysis. *An. gambiae* were placed on ice and their heads were separated from the thorax and abdomen. Groups of 25 heads were than stored in liquid nitrogen. For the MI group, mosquito thoraces and salivary glands were screened first under a fluorescent microscope and only the heads positive for GFP *An. gambiae* were collected. The infection was repeated in the same way on a second cohort of *An. gambiae* 1 month later. Finally, six samples of heads were analysed: MI_1, NI_1, C_1 from the first cohort and MI_2, NI_2, C_2 from the second cohort.

2.2 Preparation of protein samples and labelling

For each sample, 50 frozen heads were crushed in a micro tissue grinder. The crushed heads were then suspended for 1 h at 20°C in 0.5 mL of an extraction/precipitation buffer (10% TCA in acetone, 0.07% β-mercaptoethanol). The samples were centrifuged at $30\,000 \times g$ for 15 min at 3°C. Once the supernatants were removed, samples were suspended in a wash buffer (0.07% β-mercaptoethanol in acetone). The wash step was repeated three times. After that, samples were suspended in 40 μL of a solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, 0.5% Triton X100, adjusted to pH 8.5) overnight at room temperature with agitation. Following centrifugation (15 min at $30\,000 \times g$), the protein content was determined using the 2D-Quant Kit (GE Healthcare).

Proteins were labelled according to the Ettan DIGE minimal labelling protocol (GE Healthcare). For each sample, 20 μg of protein was labelled with 150 pmole of

CyDye™. To determine and exclude non-specific labelling between groups (C, NI, MI) in both cohorts ($_{1,2}$), a 'forward' (test proteins, *i.e.* $MI_{1,2}$, labelled with Cy3, control proteins, *i.e.* $C_{1,2}$ and $NI_{1,2}$, labelled with Cy5) and a 'reverse' (test proteins labelled with Cy5, control proteins labelled with Cy3) labelling was done (Table S1, Supporting Information). An internal standard consisting of aliquot of all six samples (*i.e.* MI_1, MI_2, NI_1, NI_2, C_1, and C_2) was labelled with Cy2. The Cy2 internal standard was used to normalise protein abundances across gels and to control for gel-to-gel variation [21].

2.3 Protein separation by 2-DE

For IEF, an internal standard labelled with Cy2, one sample labelled with Cy3 (*e.g.* the MI sample) and another sample labelled with Cy5 (*e.g.* the NI sample) were mixed in a rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X100, 30 mM Tris·pH 8.8, 1.4 µL Destreak Reagent (GE Healthcare) and 0.75% IPG buffer pH 3–10). The solubilized proteins were loaded onto 24-cm IPG strips, pH 3–10, NL (GE Healthcare). Following 14 h of passive rehydration, IEF was performed using an IPGphor™ apparatus (GE Healthcare) as follows: 3 h at 100 V, 3 h gradient to 1000 V, 4 h gradient to 8000 V and 8000 V constant to reach a total of 50 000 Vh. After IEF, the strips were incubated for 15 min in equilibration buffer (6 M urea, 300 mM Tris pH 8.8, 0.2% SDS, 30% glycerol, 1% DTT) followed by 15 min in equilibration buffer where DTT was replaced with 2.5% iodoacetamide. The equilibrated IPG strips were placed on top of an SDS-polyacrylamide gel (15% acrylamide) and sealed with 1% agarose. The electrophoresis was performed using an Ettan Dalt Twelve (GE Healthcare) at 17°C and 17 mA/gel.

2.4 Gel imaging, image analysis and statistics

2-D DIGE gels were scanned using a Typhoon 9400 set according to the manufacturers' instructions (GE Healthcare). Images were pre-processed using the ImageQuantTM software (GE Healthcare). Intra-gel spot detection and inter-gel matching were performed using the Differential In-gel Analysis (DIA) mode and Biological Variation Analysis (BVA) mode of "DeCyder" software (GE Healthcare), respectively. Protein spot volumes were normalised to the internal standard (see Section 2.3). One-way analysis of variance (ANOVA) was used to reveal significant protein expression differences among the six samples (MI_1, NI_1, C_1, MI_2, NI_2, C_2). Thereafter, significantly under-/over-expressed proteins were identified by multiple comparisons using the Student's *t*-test. Spots of interest, *i.e.* protein spots present on all 27 images (3 dyes × 9 gels, Table S1, Supporting Information) and differentially expressed in the MI group were identified using MS. The identity of some candidate protein spots was confirmed by MS/MS.

2.5 Protein identification by MS and MS/MS

After 2-D DIGE, two 2-DE gels were run with 400 µg of a mix of protein from the different samples, and stained with CBB. Spots of interest were localised on the gel by comparing the CBB-stained spot pattern with the 2-D DIGE protein pattern. Spots were then excised manually in a laminar flow hood to prevent contamination with keratin. Enzymatic digestion was performed automatically (Tecan freedom evo® proteomics). MS analysis was performed using an UltraFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in 'reflectron' mode with an accelerating voltage of 26 kV and a delayed extraction of 50 ns. Mass spectra were acquired in an automatic mode using the AutoXecute module of Flexcontrol (Bruker Daltonics). Spectra were analysed using the FlexAnalysis software (Bruker Daltonics) and calibrated internally with the auto-proteolysis peptides of trypsin (*m/z* 842.51, 1045.56, 2211.10). Proteins were identified in the *Anopheles* genome database through PMF by using the program MASCOT (Matrix Science, London). Taking into consideration the possibility of molecular crosstalk between the mosquito and the malarial parasite *via* the synthesis of mimetic proteins or of host contamination, a parsimony search with all categories of the host–parasite system was also performed. One missed cleavage *per* peptide was allowed, and an initial mass tolerance of 25 ppm was used in all searches. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionines was fixed as a variable modifications. MS/MS analysis was performed using the same mass spectrometer in an LIFTTM mode, using a 0.5 to 1% range for the ion mass selector window. Proteins were identified in the Metazoa database with MASCOT. A mass deviation of 25 ppm was allowed for database interrogation for parent mass and 0.5 Da for fragment ions.

3 Results

3.1 Analysis of gels

After detection (around 2000 spots detected *per* gel), image gels were "cleaned off" for speckling and artefact spots. The resulting number of protein spots analysed was 1400. In our study, 2-D DIGE was used to reveal mainly soluble proteins in relatively high abundance and in a specific pH range (*i.e.* 3–10). Under these restrictions, 35 protein spots were detected as being differentially expressed (Table S2, Supporting Information). Twelve protein spots were up- or down-regulated in the MI group in both cohorts (*i.e.* MI_1 and MI_2) (Fig. 1: spots Ma-Ml and Fig. S1, Supporting Information): these particular protein spots are characteristic of the mature infection in the head of *Anopheles* and are thus potentially involved in behavioural modifications. One protein spot was shown to be down-regulated in the NI group in both cohorts (*i.e.* NI_1 and NI_2) (Fig. 1: spot Na and Fig. S1, Supporting Information).

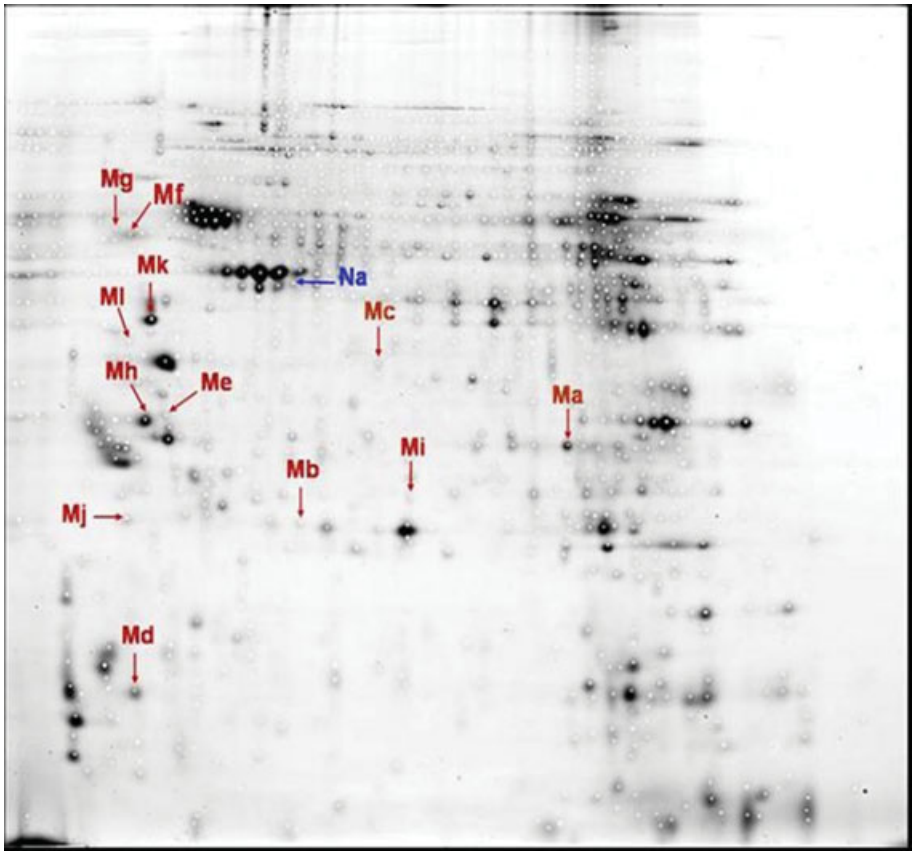

Figure 1. Protein spots differentially expressed between the groups are indicated by an arrow on this 2-D DIGE gel. Protein spots up-/down-regulated in the head of sporozoite-infected mosquitoes (MI group) in both cohorts are indicated in red (spots Ma-Ml). The only protein spot (spot Na) down-regulated in the head of *Plasmodium* mutant (2.33) is indicted in blue.

3.2 Identification

The 12 protein spots of interest (spots Ma-Ml) and the Na protein spot were identified by PMF (see Table 1). The PMF spectra are given in Fig. S2, Supporting Information. MS/MS was used to confirm some protein identities (see Table S3 and Fig. S3, Supporting Information). The 13 proteins identified represent 12 unique genes. One identified protein (Q7PQP1 (Mf, Mg)) was indeed detected in more than one spot, suggesting different isoforms and/or PTM (Fig. 1 and Table 1).

The 13 identified proteins appearing with altered levels in the heads of infected mosquitoes in both cohorts can be functionally classified as metabolic, synaptic, molecular chaperone, signalling and cytoskeletal proteins (Table 2).

3.2.1 Metabolism

Of the five metabolic proteins identified, four were up-regulated in the MI group (Ma-Md, respectively from the PGAM, ADK, HAD-SF_hydro_IIA, COX5A families, Table 1), whereas one was weakly down-regulated in the NI group (Na from the ATP-grasp family). The protein functions are indicated in Table 2.

3.2.2 Synaptic transmission

The protein belonging to the BSD family domain (Mf, Mg, two isoforms or PTMs) is a homolog of the conserved *Drosophila* synapse-associated protein SAP47 [22, 23]. Synapse-associated proteins are expressed specifically in neurons and act as important molecular elements of the nervous system [22, 24]. Here, we show that this protein is down-regulated in the head of sporozoite-infected *Anopheles* (Table 2).

3.2.3 Heat shock response

One identified molecular chaperone (Mi, Hsp20 of the small heat shock protein family) is up-regulated in the MI group (Table 2).

3.2.4 Signalling

Calmodulin (Mj, efhand family), the only signalling protein identified, was strongly up-regulated in the MI group (Table 2). The signalling role of calmodulin is intimately connected with the prevailing intracellular concentration of free Ca^{2+} ions. In the insect CNS, calmodulin is involved in nitric oxide (NO) synthesis [27–29]. NO is generated in

Table 1. MS identification of protein spots up-/down-regulated in the head of sporozoite-infected mosquitoes (MI group) in both cohorts (spots Ma-MI) or in the head of *Plasmodium* mutant (2.33) (spot Na down-regulated)

Spot no.	(Access. no.) Name of protein[a)]	(exp. M_r; exp. p*I*)/ (th. M_r ; th. p*I*)	MASCOT score[b)]	Coverage (%)
Ma	(Q7PXI5) ENSANGP00000015800	(29; 6.33)/ (29; 6.38)	122	58
Mb	(Q7QGG2) ENSANGP00000015199	(23; 5.61)/ (26; 5.71)	65	29
Mc	(Q7QEP8) ENSANGP00000019927 (Fragment)	(38; 6.12)/ (34; 5.84)	128	52
Md	(Q7PSR9) ENSANGP00000022070 (Fragment)	(14; 4.46)/ (17; 4.99)	63	28
Me	(Q7PKX4) ENSANGP00000022447	(32; 4.63)/ (30; 4.64)	117	41
Mf	(Q7PQP1) ENSANGP00000013960 (Fragment)	(56; 4.29)/ (51; 4.53)	82	24
Mg	(Q7PQP1) ENSANGP00000013960 (Fragment)	(56; 4.19)/ (51; 4.53)	69	25
Mh	(Q7PX08) ENSANGP00000012072	(29; 4.7)/ (29; 4.56)	131	50
Mi	(Q7PT53) ENSANGP00000018891	(25; 6.34)/ (22; 6.24)	86	47
Mj	(Q5TQ67) ENSANGP00000025629	(23; 4.36)/ (22; 4.52)	63	42
Mk	(Q7PY91) ENSANGP00000023232	(33; 4.51)/ (24; 4.44)	55	27
Ml	(Q7PS96) ENSANGP00000015145	(40; 4.34)/ (36; 4.43)	65	21
Na	(Q7QA95) ENSANGP00000013032 (Fragment)	(49; 5.63)/ (44; 5.48)	112	40

a) 'Access. no.' refers to accession number in Swiss-Prot and TrEMBL protein databases (http://www.expasy.org/)
b) 'MASCOT scores' >50 indicate significant identity or extensive homology ($P \leq 0.05$)
Q7PQP1 is a protein detected in more than one spot (Mf and Mg) (may be indicative of either different isoforms and/or PTMs)

neurones by Ca^{2+}/calmodulin-activated nitric oxide synthase (NOs), which catalyses the conversion of L-arginine and oxygen to NO and citrulline (Table 2).

3.2.5 Cytoskeleton

Two identified cytoskeletal tropomyosins (Me, Mk) were up-regulated in the MI group (Table 2).

3.2.6 Miscellaneous

Two proteins, up-regulated in the head of sporozoite infected mosquitoes, have miscellaneous functions and thus have not been functionally classified (Mh, a 14-3-3 protein and Ml, an annexin, Table 2).

4 Discussion

Very little is known about the CNS functioning of mosquitoes infected with malaria parasites. Exploring the infected insect vector's head proteome gives us the opportunity to elucidate the proximate mechanisms involved in behavioural manipulation. Using 2-D DIGE coupled with MS, the present study provides evidence that *P. berghei* induces alteration in the head proteome of *A. gambiae*.

Four metabolic enzymes were up-regulated in the MI group suggesting an increased energy metabolism in the head of sporozoite-infected mosquitoes. A modification of metabolic enzyme activity could modify the production of ATP and consequently be detrimental to normal brain and neuronal function [34–36]. In the CNS of vertebrates, ATP is not only the energy source but can also act directly as a neuromodulator at purinergic synapses and as a factor with the potential to regulate: (i) neural development and plasticity; (ii) proliferation and apoptosis of glial and brain capillary endothelial cells; and (iii) the response of the nervous system to disease processes [37, 38]. Evidence concerning these roles in the insect CNS is lacking, but readers are referred to a publication by Magazanik and Fedorova [39]. Vertebrates and invertebrates share a common ancestral CNS [40, 41] and neuromodulatory actions of ATP probably precede, in evolutionary terms, neuropeptides as an intercellular messenger [42]. Consequently, a neuromodulatory role of ATP is likely to also be involved in the insect CNS.

A recent study of the energetic budget of *A. stephensi* infected with *P. chabaudi* [43] revealed that the body of mosquitoes infected with the oocyst stage contained up to 50% more glucose than control mosquitoes. The authors demonstrated that this phenomenon is parasite-mediated and results from increased glucose uptake from blood and not from a reallocation of resources. The authors conclude by calling for more studies to investigate the physiological role of glucose in the *Plasmodium*-mosquito interaction. Taken together with our results, these experiments suggest the possibility that prior to becoming infective to the vertebrate host during immature infection, *Plasmodium* manipulates the sugar uptake of the vector [43] and then, once reaching the infective stage, manipulates the vector's behaviour through altered normal neuronal functions, by inducing in the head increased glucose oxidation.

Several proteins identified here are known to play critical roles in the CNS and in neuronal functioning. For instance, the down-regulation of the synapse-associated protein (see

Table 2. Functions of up-/down-regulated proteins

Spot no.	Up-/down-regulation [a]	Family[b]	Functional class[c]		Biological processes[c]
Ma	(+)	PGAM	Metabolism	Glucose oxidation pathway	Phosphoglycerate mutase, a glycolytic enzyme, is responsible for the interconversion of 3- phosphoglycerate to 2-phosphoglycerate
Na	(−)	ATP-grasp			The subunit family represented here is primarily found in succinyl-CoA synthetases, an enzyme that catalyse the conversion of succinyl coA to succinate in Krebs cycle
Md	(+++)	COX5A			Complex IV of mitochondrial respiratory chain (subunit Va) is involved in oxidative phosphorylation
Mb	(+++)	ADK		other	Adenylate kinase is a phosphotransferase enzyme that catalyses the production of ATP from ADP
Mc	(+++)	HAD-SF_hydro_IIA			This subfamily contains phosphoglycolate phosphatase (in photosynthetic organisms it catalyses the dephosphorylation of 2-phosphoglycolate), and 4-nitrophenyl phosphatase (catalyses the hydrolysis of nitrophenyl phosphates to nitrophenols). The role of these proteins in animals remains largely unknown
Mf, Mg	(−); (−)	BSD	Synaptic transmission		Synapse-associated protein, the homolog of *Drosophila* SAP47 [22–24]
Mi	(++)	HSP20	Molecular chaperone		Molecular chaperone prevent the misfolding of proteins during cell stress. Hsp20 protect cardiomyocytes from apoptosis [25] and prevent the β-amyloid (Aβ) peptide fibril formation and toxicity in human [26].
Mj	(+++)	Efhand	Signalling		Ca^{2+} dependent regulation of wide variety of cellular events [27], activation of nitric oxide synthase [28, 29].
Me Mk	(++) (+)	Tropomyosin	Cytoskeleton		Rod-shaped, coiled-coil protein that binds along the side of the actin filament. In muscle cells, tropomyosin helps mediate the signal for contraction, controlling access of myosin heads to the actin filament. In non-muscle cells, tropomyosin prevents the dissociation of actin subunits from microfilaments and increases the stability and physical strength of microfilaments [30].
Mh	(+++)	14-3-3	Miscellaneous		Multifunctional regulators participating in neurotransmitter synthesis, cell cycle, transcriptional control, signal transduction, intracellular trafficking and regulation of ions channel [31, 32].
Ml	(+++)	Annexin			Multiple cellular processes such as structural organization of the cell, intra-cellular signalling by enzyme modulation, ion flux, growth control and atypical calcium channel [33]

a) The scores of up-/down-regulation are derived from the average ratio of the normalised spot volumes in S1: + an up-regulation of 10–20%, ++ 20–30%, +++ >30%, – down-regulation 10–20%, – 20–30%, — > 30%

b) 'Family' indicates the protein family or domain according to the PFAM database of Sanger Institute (http://www.sanger.ac.uk/Software/Pfam/) or the InterPro database of the European Bioinformatics Institute (http://www.ebi.ac.uk/interpro/)

c) Association of each protein with functional class and biological processes have been determined by gene ontology database (http://www.geneontology.org/) and literature search

Mf, Mg in Table 2) in the head of sporozoite-infected mosquitoes indicates a possible alteration in neuronal functioning [24]. In addition, since tropomyosins are involved in neuronal morphogenesis [30, 44], the variation in cytoarchitectural proteins shown here (spots Me and Mk) may trigger structural changes in the *Anopheles* CNS and hence may warrant further investigations into the effect of an altered cytoskeleton. The up-regulation of the 14-3-3 protein may also have important consequences on neuronal integrity. In *Drosophila*, mutants of 14-3-3 gene exhibit defects in olfactory learning, memory and physiological neuroplasticity at the neuromuscular junction [45]. The up-regulation of calmodulin (signalling protein, spot Mj) may indicate an alteration in NO synthesis. NO is a neuromediator also involved in immunological reactions. In the insect brain, it is known to influence a wide variety of processes such as the formation of olfactory memory and vision as well as neuronal development [46]. In this con-

text, our findings indicate a possible alteration in the neuronal integrity of the CNS in sporozoite-infected *Anopheles*.

Interestingly, two proteins revealed here have been demonstrated to be involved in behavioural modifications in other host–parasite systems. Tropomyosin has been shown to be involved in the behavioural manipulation of crustacean gammarids by acanthocephalans [16], while PGAM are involved in cricket behavioural manipulation induced by hairworms [15]. The comparison of proteins involved in behavioural modifications in different host–parasite systems raises the question of molecular convergence in manipulation processes. In effect, do phylogenetically distant parasites use the same proximate mechanisms to alter the behaviour of their host, hosts belonging to different taxa? Our findings provide examples of molecular convergence and seem to indicate that the "*jeu des possibles*" to manipulate the behaviour of a host is limited. To investigate this further, we suggest additional studies on a broader range of host–parasite systems.

In conclusion, many studies report behavioural modifications in malaria-infected mosquitoes. This work is the first to explore the possibility that the vector head proteome is altered during infection with the *Plasmodium* parasite. We provide empirical evidences supporting the view that the malaria parasite does indeed cause modifications to the head proteome of its insect host. Several identified proteins showing altered levels in sporozoite-infected mosquitoes provide an explanation for the observed behavioural alterations. These proteins can be grouped into five main pathways: metabolic, signalling, cytoskeleton, heat shock response and synaptic transmission. We suggest that the altered ATP synthesis pathways could be responsible for the behavioural modifications: (i) directly through the potential roles of ATP as neuromodulators and/or (ii) indirectly through modifications of cellular energy metabolic mechanisms that subsequently have detrimental effects on neuronal cells. In addition, we suggest that the 14-3-3 protein and the homolog of the *Drosophila* synapse-associated protein SAP47, with their central roles in neuron regulation, are potentially involved in behavioural modifications. Molecular findings that head proteome alteration occurs mainly during mature infection (*i.e.* 12 proteins out of 13 are altered only in the head of sporozoite-infected mosquitoes, see Table 1), support previous behavioural studies showing that modifications take place only when the parasites reach the infective stage [4, 7, 9]. However, a note of caution is warranted: to date, this proteomic study has identified correlations between vector behaviour and putative molecular mechanisms to explain the behaviour. To the best of our knowledge, there is no quantitative assay that can prove whether a specific gene(s) can alter such vector behaviour. Our data pave the way for future investigations on the *Anopheles* CNS alteration during infection by malaria parasites. The challenge will now be to identify exactly how the candidate proteins interfere with normal neuronal functioning in order to determine the key points responsible for behavioural manipulation.

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