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Trypanosoma brucei brucei induces alteration in the head proteome of the tsetse fly vector *Glossina palpalis gambiensis*

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Abstract

Parasitic manipulations of host behaviour are known from a wide range of host–parasite associations. However, the understanding of these phenomena is far from complete and detailed investigation of their proximate causes is needed. Many studies report behavioural modifications, such as altered feeding rates in tsetse fly (*Glossina*) infected with the mature transmissible stage (i.e. metacyclic) of the trypanosomes. Here, bidimensional (2D) gel electrophoresis and mass spectrometry were employed to analyse and compare the head proteome between four *Glossina palpalis gambiensis* categories (uninfected, refractory, mature infection, immature infection). Twenty-four protein spots specifically present or absent in the head of metacyclic-infected flies were observed. These protein spots were subsequently identified and functionally classified as glycolitic, neurotransmiter synthesis, signalling, molecular chaperone and transcriptional regulation proteins. Our results indicate altered energy metabolism in the head of metacyclic-infected tsetse flies. Some of the proteins identified, such as casein kinase 2 and jun kinase have previously been shown to play critical roles in apoptosis in insect neurones. In addition, we found two pyridoxal-dependent decarboxylases (dopa decarboxylase and alpha methyldopa hypersensitive protein), suggesting a modification of serotonin and/or dopamine in the brain of metacyclic-infected tsetse flies. Our data pave the way for future investigation of the alteration of the glossina central nervous system during infection by trypanosomes.

Keywords: *Glossina palpalis gambiensis*, *Trypanosoma brucei brucei*, behavioural changes, proteomics, mass spectrometry, MALDI/ToF, host-parasite associations.

Introduction

Host behavioural changes induced by parasites that increase the likelihood of parasite transmission have long captured the interest of parasitologists and behavioural ecologists (Moore, 2002; Thomas *et al.*, 2005). For instance, in parasite–insect vector systems, several studies support the idea that parasites manipulate the behaviour of their vectors, such as feeding behaviour, in a way that increases the contact with the vertebrate host and hence favours parasite transmission (see Molyneux & Jefferies, 1986 and Hurd, 2003). Although there are many other impressive examples of host manipulation by parasites (Moore, 2002), little is known about the proximate mechanisms underlying this intriguing parasite strategy.

In parasite–vector associations, two main mechanisms have been proposed to explain the increased feeding rate of infected bloodsucking insects. First, parasites and insect vectors may compete for metabolites in the ingested blood, and resource depletion therefore leads to new feeding attempts by the insect (Schaub, 1992; Adamo, 1997). Second, parasites may interfere in the ingestion process of the insect by, for example, obscuring phagoreceptors, blocking the foregut and reducing apyrase activity in salivary glands. These mechanisms seem to impair the vector's ability to fully engorge and therefore induce them to bite the vertebrate host several times (Molyneux & Jefferies, 1986; Hurd 2003).

In this study we focused on an insect–trypanosomid system, the tsetse fly vector *Glossina palpalis gambiensis* and the protozoan parasite *Trypanosoma brucei brucei*, an endemic association of West Africa. *Glossina p. gambiensis* is a strictly haematophagous and diurnal species. These

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flies are found in dense, wet equatorial rain forest and in local patches of dense vegetation along the banks of rivers and lakes in arid regions. *Trypanosoma b. brucei* is the causative agent of Nagana, a serious animal parasitic disease in sub-Saharan African countries. The life cycle of this trypanosome involves several different stages in the insect vector and the mammalian host. The parasite multiplies as procyclic forms in the midgut of the insect and the infection of the mammalian host occurs when the insect vector containing the metacyclic stages in its salivary glands feeds upon the host.

In tsetse flies parasitized with trypanosomes, Jenni *et al.* (1980) showed that *Glossina morsitans morsitans* infected with *T. brucei* probed three times more often and fed more voraciously than uninfected flies. Working on *G. m. morsitans* infected with *T. congolense*, Roberts (1981) reported that infected flies probed significantly more frequently and took longer to engorge than uninfected flies. The increased probing may be the result of parasites physically interfering with phagoreceptors in the tsetse fly labrum (Molyneux *et al.*, 1979; Jenni *et al.*, 1980; Livesey *et al.*, 1980, Thevenaz & Hecker, 1980). However, such findings have not been confirmed by studies performed by Moloo's group (Moloo, 1983; Moloo & Dar, 1985; Makumi & Moloo, 1991).

The central nervous system (CNS) functions to convert patterns of activity in sensory receptors into patterns of muscle activity that constitute appropriate behaviour. Thus, any changes in behaviour must have a molecular basis in the CNS (Adamo, 1997, 2002; Hamilton & Hurd, 2002). Exploring this molecular basis will undoubtedly help in elucidating just how pathogens manipulate the behaviour of their insect vector (Lefèvre *et al.*, 2006). As part of our efforts to gain a broader understanding of the proximate causes of the behavioural changes observed in infected tsetse flies, and as a first step toward deciphering the brain molecular mechanisms involved, we investigated the brain proteome of metacyclic-infected *Glossina*. Proteomics, which allows investigation of the translation of genomic information, offers an approach to studying the global changes in protein expression of the insect host CNS caused by parasites (Biron *et al.*, 2005a, 2006; Ponton *et al.*, 2006; Lefèvre *et al.*, 2007). Here, we used such an approach on the *G. p. gambiensis–T. b. brucei* system to elucidate molecular mechanisms in the brain that underlie behavioural modifications.

Using bidimensional gel electrophoresis (2-DE) coupled with mass spectrometry, the current study aimed to compare the CNS proteome in four groups of *Glossina*: (1) with mature infection (M): i.e. the parasite is present in the midgut and in the salivary glands, characterising infective flies; (2) with immature infection (I): i.e. the parasite is only localised in the midgut; (3) refractory (R): i.e. the parasite was eliminated in the days following the infective bloodmeal; and (4) uninfected (C): i.e. flies fed with uninfective bloodmeal. The I and R groups were used as secondary controls to exclude proteins that are nonspecific to mature infections and hence unlikely to be linked with behavioural changes (Biron *et al.*, 2005b). More specifically, we address the following questions: (1) Do the trypanosomes modify the brain proteome of the insect vector? (2) If yes, what is the function of the proteins differentially expressed in the brain of mature infected flies and hence what are the molecular pathways thus altered?

(A)

	C	M	I	R
C	–	639	631	666
MI	0.1	–	652	651
PI	0.104	0.044	–	649
R	0.080	0.073	0.068	–

(B)

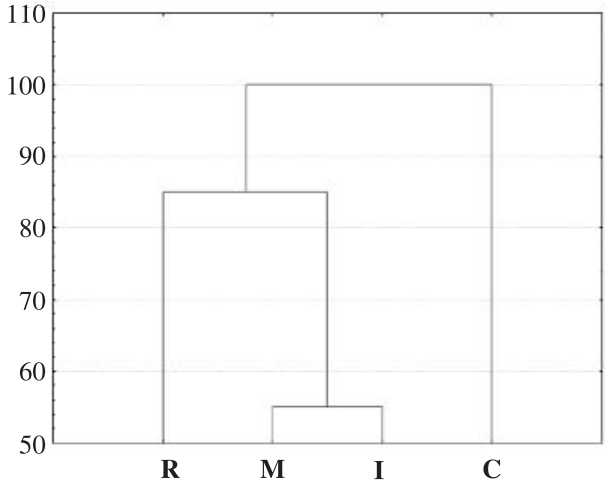

Figure 1. (A) Host proteome response: number of common protein spots (above diagonal) and proteome distances (below diagonal) between categories: Control (C) (Uninfected flies), Mature infection (M), Immature infection (I) and Refractory flies (R). (B) Classification of 2D gels resulting from the proteome distance among the four fly categories.

Results

Mortality and infection rates

The mortality rate was 20% for the uninfected group (C; $n = 20$) and 18.9% for the infected groups (M + I + R; $n = 159$). The rate of midgut infection was 13% and the rate of mature infection in the salivary glands was 5.2%.

Heuristic classification of 2-DE gels

Figure 1 indicates the number of common protein spots resolved along with the proteome distances among the four head categories (Nei & Li, 1979). The qualitative data (presence/absence of protein spots) were analysed with a

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Table 1. Number of protein spots present or absent (not detected) during host-parasite interaction. Control (uninfected flies) (C), mature infection (M), immature infection (I), refractory flies (R)

Biological interpretation	C	M	I	R	Number of protein spots
always expressed (common protein spots)	×	×	×	×	613
Mature infection (behavioural modifications)		×			12
	×		×	×	12
Resistance				×	24
	×	×	×		5
Parasite contact		×	×	×	24
	×				50
Immature infection			×		11
	×	×		×	11
Successful infection		×	×		10
	×			×	30
Unknown	×	×			10
	×		×		1
		×		×	3
Total					816

Note: X: protein spots present; blank: protein spots absent (not detected).

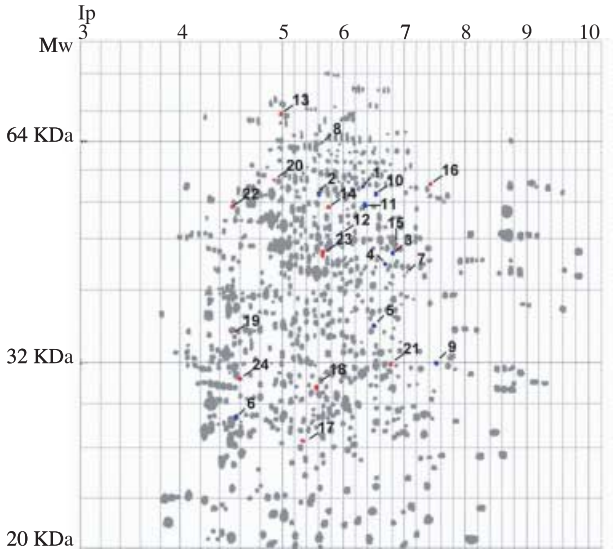

Figure 2. Two-dimensional synthetic gel showing the head proteome of *Glossina palpalis gambiensis* in response to infection by *Trypanosoma brucei brucei*. • Common and 'nonspecific' protein spots. • Proteins specifically present in the head proteome of mature infection (M) flies. ● Common and 'non specific' protein spots. ● Proteins specifically present in the head proteome of mature infection (M) flies. ● Proteins specifically missing in the head proteome of M flies.

phenetic study by calculating the 'proteome distance'. The dendogram constructed (Fig. 1B) indicates that the categories of flies fed with an infected bloodmeal (i.e. M, I and R) join together, whereas the C category is isolated. Furthermore, among the categories of flies fed with an infected bloodmeal, the two infected (M and I) join together, whereas the R category is isolated.

Analysis of 2-DE gels

Table 1 shows the number of protein spots present or absent (i.e. not detectable: break-even point of detection) in the four categories. Six hundred and thirteen protein spots were common to the four categories whilst 24 were specifically present or absent from the M category. These 24 spots characterize the mature infection in the head of *Glossina* and are thus potentially involved in behavioural modifications. In addition, protein spots specific to the three other categories were revealed (see Table 1). Figure 2 shows the synthetic gel and highlights the 24 protein spots of interest (i.e. specifically present or absent in M) as well as common and protein spots that are nonspecific to mature infection.

Identification

To refine our studies on the molecular mechanisms involved in the modification of host behaviour, we attempted to identify the 24 protein spots of interest in available online SwissProt/TrEMBL protein database (http://expasy.org/sprot/). Of the 24 protein spots of interest, 17 were identified through peptide mass fingerprinting. The results are summarized in Table 2. The identified proteins can be functionally classified as glycolytic, neurotransmitter synthesis, molecular chaperone, signalling and transcriptional regulatory proteins (see Table 2). One protein (Table 2, spot 17) has miscellaneous functions whilst four proteins have unknown functions and have thus not been functionally classified (Table 2, spots 4, 5, 9 and 23).

Glycolysis. Three glycolytic enzymes with altered levels in the head of flies with mature infection were found: enolase was present in the M group, whereas two glucose-6-phosphate 1-dehydrogenases were specifically absent in the M group. The protein functions are indicated in Table 2.

Neurotransmitter synthesis. Two neurotransmitter enzymes were found: dopa decarboxylase (DDC; spot 11, specifically absent in M group) and alpha methyldopa hypersensitive protein (spot 14, specifically present in M group). Both proteins are pyridoxal-dependent decarboxylases. DDC, also known as aromatic L-amino decarboxylase, catalyses the final step (decarboxylation) in the synthesis of dopamine and serotonin. The α-methyldopa hypersensitive gene in *Drosophila* arose from DDC duplication, and is closely linked to DDC (Eveleth & Marsh, 1986).

Signalling. Five signalling proteins were found to be differentially expressed in the M group (specifically present or absent). One protein (spot 1), containing a C2 domain, was absent in the M group. In contrast, two protein kinases [spot 15, jun kinase (JNK) and spot 21, casein kinase 2 (CK2)] were present in the M group. Lastly, two proteins containing a Mastermind (MAM) domain (spots 2, 6) were identified as absent in the M group. Protein functions are given in Table 2.

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Table 2. Mass spectrometric identification of protein spots present or absent in the head of flies with mature infection (M category) and functions of the identified proteins

Spot no.*	Presence (P) Absence (A) in M group	(Access no.)†/Name of protein	(exp Mw-exp pI)/ (thMw-thIp)	Coverage (%) (*P*-value)	Domain, Family‡	Functional class§	Description and biological processes§
7	A	(Q5TUJ4) ENSANGP00000028421 (Fragment)	(40;7.07)/(36;6.82)	23 (2.5e-6)	G6PD_C	glycolysis	Glucose-6-phosphate 1-dehydrogenase catalyses the first step in the pentose
12	A	(Q7QLH1) ENSANGP00000012074 (Fragment)	(46;5.98)/(55;6.48)	25 (7.5e-7)			pathway, i.e. the conversion of glucose-6-phosphate to gluconolactone 6-phosphate in the presence of NADP, producing NADPH.
18	P	(Q32ZR3) Enolase (Fragment)	(30;5.59)/(24;5.27)	24 (3.1e-5)	Enolase		Enolase catalyses the interconversion of 2-phosphoglycerate and phosphoenolpyruvate in the pentose pathway.
11	A	(Q402L7) Dopa decarboxylase	(50;6.38)/(54;6.05)	16 (1.9e-7)	Pyridoxal_deC	Neurotransmitter synthesis	This protein family catalyses the decarboxylation
14	P	(Q8WSE8) Alpha methyldopa hypersensitive protein (Fragment)	(50;5.78)/(38;5.85)	18 (1.9e-5)			of L dopa to dopamin and L hydroxytryptophan to serotonin.
3	A	(Q9VL02) CG5395-PA (GH08677p)	(43;6.82)/(42;8.30)	19 (1.4e-5)	AAA	Molecular chaperone	A class of chaperone like ATPase involved in assembly, operation, and disassembly of protein complexes.
15	P	(O61444) Stress activated MAP kinase kinase 4	(44;6.89)/(48;7.11)	21 (4.2e-5)	Pkinase		Jun kinase (JNK) also known as stress-activated protein belongs to the MAP kinase proteins. JNK activates the transcription factor c-Jun by phosphorylating serine 63 and 73
21	P	(O76485) Casein kinase II subunit beta	(32;6.79)/(25;5.55)	24 (5.6e-4)	CK_II_beta		Regulatory beta subunit of protein casein kinase 2 (CK2), a Ser–Thr protein kinase. Catalyses the phosphorylation of many proteins required for signal transduction, cell-cycle regulation, DNA metabolism
1	A	(Q7PEL9) ENSANGP00000024306 (Fragment)	(54;6.34)/(41;6.10)	23 (2.1e-5)	C2	Signalling	Domain found in many proteins most notably protein kinase. The C2 domain is thought to be involved in calcium-dependent phospholipid binding.
2	A	(Q5ELK4) SR-CIV	(53;5.61)/(46;5.63)	20 (1.4e-6)	MAM		An extracellular domain found in many receptors.
6	A	(Q6V6Q8) CG3212 (Fragment)	(28;4.57)/(26;5.86)	23 (5.6e-6)			Proteins containing the MAM domain have a modular, receptor-like architecture comprising a signal peptide, an N-terminal extracellular domain, a single transmembrane domain and an intracellular domain. The MAM domain is thought to have an adhesive function. Spot 2 is the SR-CIV protein (class C scavenger receptor)
24	P	(Q5TMQ2) ENSANGP00000026400 (Fragment)	(31;4.61)/(26;5.58)	25 (3.0e-7)	Znf_C2H2	Transcriptional regulation	The C2H2 zinc finger is the classical zinc finger domain: the two conserved cysteines and histidines co-ordinate a zinc ion. Zinc finger motifs are nucleic acid-binding protein structures.
17	P	(Q5TTZ2) ENSANGP00000027149 (Fragment)	(26;5.35)/(24;5.31)	26 (1.3e-6)	concanavalin A-like	Miscellaneous	Members of this family are diverse, and include the lectins, glucanases, xylanases, pentraxins and calnexins There are also many Con A-like domains found in proteins involved in cell recognition and adhesion (i.e. neurexins)

Table 2. (*Continued*)

Spot no.*	Presence (P) Absence (A) in M group	(Access no.)†/Name of protein	(exp Mw-exp pI)/ (thMw-thIp)	Coverage (%) (*P*-value)	Domain, Family‡	Functional class§	Description and biological processes§
4	A	(Q9V351) CG11191-PA (GH10486p)	(41;6.7)/(38;5.85)	26 (3.6e-5)			
5	A	(Q7QB97) ENSANGP00000020477	(35;6.52)/(29;6.65)	21 (3.4e-6)		Unknown	
9	A	(Q9VS59) CG8580-PA, isoform A	(32;7.54)/(22;9.21)	33 (1.7e-8)			
23	P	(Q6V4B5) IKKgamma (Fragment)	(43;5.69)/(31;4.79)	18 (3.5e-5)			

Note: *'Spot no.' refers to protein spots on 2D gel in Fig. 2. †'Access no.' refers to the accession number in the SWISS-PROT and TrEMBL protein databases (http://www.expasy.org/). ‡'Family' indicates the protein family or domain according to the PFAM database of the Sanger Institute (http://www.sanger.ac.uk/Software/Pfam/) or the InterPro database of the European Bioinformatics Institute (http://www.ebi.ac.uk/interpro/). Association of each protein with functional class and biological processes have been determined by the gene ontology database (http://www.geneontology.org/) and a literature search.

Heat shock response. One identified protease, which was absent in the M group, is a chaperone-like ATPase associated with the assembly, operation and disassembly of protein complexes (see Neuwald *et al.*, 1999 for a review).

Regulation of gene expression. Many proteins, most notably transcriptional regulators, contain multiple 'Zinc finger' domains that are nucleic acid-binding protein structures. These transcription factors, which can behave as activators or repressors, bind to sequences present in the promoters of genes encoding, for example, cell-cycle regulators, MAP kinases, regulatory GTPases, histones, enzymes involved in DNA synthesis and growth factors. Here we show that as regulatory protein containing a 'Zinc Finger' domain (spot 24) is specifically present in the M group.

Miscellaneous: As mentioned earlier, one protein present in the M group has miscellaneous functions and was thus not functionally classified (spot 17, Table 2).

Discussion

To date, nothing has been published concerning the CNS functioning of tsetse flies infected with trypanosomes. The present study is the first to explore the possibility that molecular changes operate in the brain of tsetse flies during infection with trypanosomes. Using 2-DE coupled with mass spectrometry, we show that proteomics tools are sensitive enough to detect intraspecific proteome variations linked to parasitic status and even to parasite stage (i.e. mature versus immature infection). Our proteomics analysis revealed proteins with an altered level in the head of metacyclic infected flies and thus that are potentially involved in behavioural manipulation. These candidate proteins are known to play crucial roles in metabolism, cellular signalling pathways, neurotransmitter synthesis, transcriptional regulation and in heat shock responses.

Altered energy metabolism

Three glycolytic enzymes have been found to be differentially expressed (present or absent) suggesting alterations in energy metabolism. Glucose is the major substrate utilized for brain metabolism (Hertz & Dienel, 2002; Ikemoto *et al.*, 2003) and the glycolytic pathway is essential for maintaining normal brain and neuronal functions in vertebrates and invertebrates (Ames, 2000; Ikemoto *et al.*, 2003). For instance, alteration of brain metabolic activity is one of the causes of numerous neurological disorders and glycolytic enzymes described here have been shown to be involved in schizophrenia (Prabakaran *et al.*, 2004) and Alzheimer's disease in humans (Bigl *et al.*, 1999). Beyond the potential indirect role of altered metabolism on behaviour, an alternative hypothesis is that the parasite induces a nutritional stress associated with a global metabolism disorder in several

tissues (i.e. not only in the brain) that leads secondarily to new feeding attempts. Alteration in energy metabolism has been shown recently in another insect vector–pathogen system, *Anopheles* mosquito–*Plasmodium* (Lefèvre *et al.*, 2007). Taken together, these findings provide substantial evidence of metabolic disturbance in the brain of infected vectors and warrant further investigation into the effects of this phenomenon on vector feeding behaviour.

Altered signal transduction

Several proteins described here contain domains with functional roles in signal transduction. Among these, two proteins with a MAM domain have been detected (Beckmann & Bork, 1993). Such proteins include meprin (metalloendopeptidase activity), neuropilin (cell-surface glycoprotein involved in neuronal development, Fujisawa *et al.*, 1997) and protein tyrosine phosphatase (development of nervous system; see Arregui *et al.*, 2000 for review). In addition, a protein containing a C2 domain has been shown. The C2 domain is a Ca^{2+}-dependent membrane-targeting module found in many cellular proteins involved in signal transduction or membrane trafficking. C2 domains have been identified in a variety of proteins, including protein kinase, synaptotagmin, phospholipases, GTPase-activating proteins and rabphilin. Lastly, two protein kinases (CK2 and JNK) have been revealed. CK2 is a conserved protein kinase and interacts with several signalling pathways. Implication of CK2 in neoplasia, apoptosis, virus infection and insect nervous system development is supported by increasing amounts of data (Ahmed *et al.*, 2002; Meggio & Pina, 2003; Ivanov *et al.*, 2004). JNK is activated in response to a variety of cellular stresses and is involved in apoptosis in vertebrate and insect neurons (Kawasaki *et al.*, 1999; Leppa & Bohmann, 1999; Rusconi *et al.*, 2000; Moreno *et al.*, 2002). Apoptosis or programmed cell death, the regulatory mechanism for removing unneeded cells, can be manipulated by parasites. For example, many protozoan parasites such as *Plasmodium* spp., *Toxoplasma gondii* or *Trypanosoma* spp. are able to modulate host apoptosis pathways to their own advantage (Luder *et al.*, 2001; James & Greene, 2004). Here, the specific presence of two apoptotic regulatory proteins may indicate an alteration of programmed cell death in the head of metacyclic infected flies.

Altered neurotransmitter synthesis

In the present study, we report pyridoxal-dependent decarboxylases with altered levels in the head of metacyclic infected flies. These enzymes catalyse the final step in the synthesis of dopamine (DA) and serotonin (5-HT). Behavioural changes in parasitized hosts have long been associated with changes in brain monoamine neurotransmitters. For example, changes in brain monoaminergic activity may explain behavioural manipulation of sticklebacks by tapeworm parasites (Overli *et al.*, 2001), as well as rodents infected with nematodes (Terenina *et al.*, 1997). 5-HT has been implicated in the altered phenotype of amphipod hosts infected with both acanthocephalan and trematode parasites (Helluy & Holmes, 1990; Maynard *et al.*, 1996; Helluy & Thomas, 2003; Ponton *et al.*, 2006; Tain *et al.*, 2006).

Regulation of behavioural traits

Throughout the animal kingdom, many key genes associated with behavioural traits are conserved not only in their sequences but also in their functions. The cGMP-dependent protein kinase (PKG) and G-protein-coupled receptor (GPCRs) are known to be directly involved in the genetic control of the foraging behaviour in insects. PKG is an important cell signalling protein that influences the intensity levels of the foraging behaviour (Fitzpatrick & Sokolowski, 2004; Pennisi, 2005) and GPCRs play crucial roles in odorant and gustative path of insects (Fox *et al.*, 2001). Because of this, the GPCRs are likely to be involved in the host seeking and host choice of the vector and thus, probably affect vectorial capacity (Fitzpatrick & Sokolowski, 2004). The protocol used here did not permit the study of such membrane (insoluble) proteins and we did not observe a differential expression of PKG. However, our results offer (1) new 'candidate genes' potentially involved in natural vector feeding behaviour (Fox *et al.*, 2001) and (2) new pathways potentially involved in the behavioural manipulation of vectors by pathogens.

Conclusion

We have provided evidence of *T. b. brucei*-induced alterations in the head proteome of infected tsetse flies. Several identified proteins specifically present or absent in metacyclic infected flies are good candidates to explain the insect host's alterations in behaviour. These proteins can be grouped into five main pathways: metabolic, neurotransmitter synthesis, heat-shock response, signalling and transcriptional regulation. Interestingly, *T. b. brucei* appears to alter similar biochemical pathways in the head of its tsetse fly vector to those altered in the head of the mosquito, *Anopheles gambiae* (Lefèvre *et al.*, 2007) by the malaria parasite *Plasmodium berghei* (e.g. sugar metabolisms, signal transduction, and heat shock response; see Table 2 and Lefèvre *et al.*, 2007). The comparison of proteins involved in behavioural modifications in different systems raises the question of molecular convergence in manipulation processes. Thus, do phylogenetically distant parasites use the same proximate mechanisms to alter the behaviour of their hosts? For example, the modulation of host apoptosis pathways might be a common mechanism involved in host behavioural modifications. There is no doubt that more research is needed to fully disentangle the mechanisms involved in insect vector behavioural changes induced by

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parasites. For example, it is possible that other key host or parasite proteins were missed here as a result of the limitations of the global proteomics approach used (e.g. low abundant, low molecular weight, insoluble proteins). Similarly, because our experiment was carried out using one biological treatment on only one laboratory strain, further studies would be necessary to confirm the generality of the findings. Finally, further analyses (peptide sequencing, study of protein structure, functions and interactions, RNA interference, microarrays) would also be necessary to fully understand and confirm the roles of the proteins detected here. We hope that our findings will enhance understanding of these behavioural processes and encourage new directions of study to elucidate the intimate interactions between trypanosomes and their tsetse fly vector.

Experimental procedures

Tsetse flies

The *G. p. gambiensis* used here originated from Burkina Faso and were kept in culture at the CIRAD–EMVT, Montpellier, France. *Trypanosoma b. brucei* (Sideradougou) was isolated from oxen from Sideradougou, Burkina Faso. To avoid the possible effects of sex-specific factors on the proteomic expressions, only male tsetse flies were used for the proteomics analysis. The infection of male flies was carried out following the method described in Ravel *et al.* (2003). *Trypanosoma b. brucei* stabilate was thawed at room temperature and 0.2 ml was injected intraperitoneally into five BALB/cByJ©Ico mice (Charles River, Wilmington, MA, USA). The infection was monitored by examining tail blood using a phase-contrast microscope at 400× magnification. Teneral flies of the same age were fed on the bellies of infected mice displaying levels of parasitaemia between 6 and 12×10^7 parasites/ml (determined using the matching method, Herbert & Lumsden, 1976). All the flies were then maintained by feeding on uninfected rabbit, 3 days a week, until the end of the experiment. To avoid the chance of re-infection of the flies, before each meal, the rabbit was monitored for parasites by examination of the buffy-coat (Murray *et al.*, 1977) prepared from a blood sample taken from the ear, by phase contrast microscopy at 400× magnification.

On the 40th day after the infective feed (sufficient time for the completion of *T. b. brucei* developmental cycle in *Glossina*) the flies which were still alive were dissected. Before dissection, flies were starved for 72 h to reduce partially digested blood and thus facilitate observation of the trypanosomes in the midguts. Midgut and salivary glands were examined for trypanosomes by phase contrast microscopy at 400× magnification, allowing identification of three *Glossina* groups: M, I and R. Male *G. p. gambiensis*, of the same age as the others, fed on uninfective bloodmeal constituted group C. For each of the four groups, heads of ten male flies were isolated and stored at –80 °C prior to electrophoretic testing of the head proteome.

Protein extraction

For each of the groups, heads of ten male flies were put in a 1.5 ml microcentrifuge tube at 4 °C. To recover the water-soluble proteins and to avoid the risk of contamination, each sample was rinsed three times in a 10 mM Tris–HCl solution, pH 7.4. For each species, samples were crushed using an electric mini-grinder in 40 ml of an homogenizing solution (15 M urea, 10 mM Tris–HCl, pH 7.4, 5% (v/v) b-mercaptoethanol, 2% ampholytes, pH range 5–8) in an ice bath (Bossis & Mugniéry, 1993; Marché, 2003). The concentration of each protein sample was estimated by measuring the shift of extinction of Coomasie Blue G-250 at a wavelength of 595 nm (Bradford, 1976). Protein concentration was standardized at 2 mg/ml by the addition of the required volume of the homogenizing solution. The protein samples were stored at –70 °C prior to electrophoresis separation on 2-DE.

Two-dimensional electrophoresis (2-DE)

The first dimensions were carried out with the PROTEIN Isoelectric Focusing (IEF) Cell (BioRad, Hercules, CA, USA). One (first)-dimensional BioRad immobiline dry strip gels (IPG strip) of pH 3–10 were rehydrated in passive conditions (0 V, 20 °C) for 10 min in a denaturation solution (7 M urea, 2 M thiourea, 4% Chaps, 0.24% Triton X100, 20 mM DTT, 4% carrier ampholytes pH 3–10). For each head category, 50 μg of protein was deposited per IPG strip in order to respect the linear dynamic range of the silver nitrate stain used (Patton, 2000). At least four IPG strips of pH 3–10 were run per head category. Following the passive rehydration and after recovering the IPG strips with mineral oil, an active rehydration was performed at 0–200 V for 1 h, 1000 V for 1 h and 10 000 V for 8 h, finally reaching a maximum 80 000 V/h. After isoelectric focusing (IEF), the IPG strips were incubated at room temperature for 15 min in an equilibration buffer [6 M urea, 2% (w/v) SDS, 20% (w/v) glycerol, 375 mM Tris–HCl, pH 8.8, 130 mM DTT]. A second equilibration step was performed for 20 min in the same buffer, except that DTT was replaced by 135 mM iodoacetamide. The IPG strips were rinsed with de-ionized water for 1–2 s. At least four IPG strips of pH 3–10 were run per head category. Following the two rehydration steps, the IPG strips were laid on top of the 1.5 mm vertical second-dimensional separating gel (PROTEAN II XL Cell; BioRad vertical system) (10% T, 1.5 M Tris–HCl, pH 8.8, 10% SDS, acrylamide/PDA). Electrophoresis in the second dimension was performed at 30 mA/gel for 20 min, followed by 60 mA/gel. SDS–PAGE was halted when the bromophenol blue tracking dye had reached the bottom of the gel. Gels were stained using the tetrathionate–silver nitrate technique (Shevchenko *et al.*, 1996).

Computer analyses

At least three well-replicated gels were prepared per category. Replicated gels for each treatment were compared using ImageMaster™ 2D Platinum Software Version 5.0 (Amersham Biosciences, Piscataway, NJ, USA; GeneBio, Geneva, Switzerland). The best gels obtained for each category were then used to build a 2-D synthetic gel. The selective criteria for these gels were the absence of significant distortion, a low background and good staining. The isoelectric point and molecular weight (Mw) scales of 2-D gels were determined using a protein standard kit from BioRad. The association coefficient was used for the heuristic classification: $F = 2nxy/(nx + ny)$ where nx and ny are the number of protein spots scored in categories x and y, respectively, and where nxy is the total number of protein spots shared by both categories x and y. The proteinic divergence (proteome distance) between categories in this study was 1-F (Thomas & Singh, 1992; Tastet *et al.*, 1999). We used the Statistica 5.0 software (Statsoft Inc., Tulsa, OK, USA) to perform a heuristic analysis to classify gels based on proteinic distance.

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Protein identification by Maldi-Tof mass spectrometry

Once initial analyses had revealed protein spots of potential interest, new gels were run and silver-stained following the procedure of Shevchenko *et al.* (1996). Peptide digestion and Maldi/Tof analysis were performed as per Biron *et al.* (2005c). Mass spectrometry analysis was performed using an UltraFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in a '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 using the ALDENTE software (http://www.expasy.org/tools/aldente/) in the Swiss prot and TrEMBL databases. Monoisotopic peak lists were imported into ALDENTE with the following search parameters: (1) Insect and other Insects; (2) pI ± 2.0; (3) Mw ± 20%; (4) one missed cleavage; (5) trypsine digestion; (6) carbamidomethylation as a cysteine modification; and (7) oxidation of methionine. Search tolerance was set at 1Da with a MH+ charge state (Wilkins & Williams, 1997; Ostrowski *et al.*, 2004; Barrett *et al.*, 2005). Taking into consideration the possibility of parasite protein contamination during salivary gland dissection and/or molecular cross-talk between the host and the parasite, we also performed protein searches in the protozoa species field. Proteins were retained with the highest score and significant probability values ($P < 0.05$, i.e. the probability that the observed match is not a random event). Matching peptides with missed cleavages were considered as relevant only when there were two consecutive basic residues or when arginine and lysine residues were followed by a proline or acidic residues inside the peptide amino acid sequence (Garin *et al.*, 2001; Bécamel *et al.*, 2002).

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