Tsetse salivary glycoproteins are modified with paucimannosidic *N*-glycans, are recognised by C-type lectins and bind to trypanosomes

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Running title: *Glycomics of tsetse fly saliva*

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Abstract

African sleeping sickness is caused by *Trypanosoma brucei*, a parasite transmitted by the bite of a tsetse fly. Trypanosome infection induces a severe transcriptional downregulation of tsetse genes encoding for salivary proteins, which reduces its anti-hemostatic and anti-clotting properties. To better understand trypanosome transmission and the possible role of glycans in insect bloodfeeding, we characterized the *N*-glycome of tsetse saliva glycoproteins. Tsetse salivary *N*-glycans were enzymatically released, tagged with either 2-aminobenzamide (2-AB) or procainamide, and analyzed by HILIC-UHPLC-FLR coupled online with positive-ion ESI-LC-MS/MS. We found that the *N*-glycan profiles of *T. brucei*-infected and naïve tsetse salivary glycoproteins are almost identical, consisting mainly (>50%) of highly processed $Man_3GlcNAc_2$ in addition to several other paucimannose, high mannose, and few hybrid-type glycans. In overlay assays, these sugars were differentially recognized by the C-type lectins mannose receptor and DC-SIGN. We also show that salivary glycoproteins bind strongly to the surface of transmissible metacyclic trypanosomes. We suggest that although the repertoire of tsetse salivary *N*-glycans does not change during a trypanosome infection, the interactions with mannosylated glycoproteins may influence parasite transmission into the vertebrate host.

Introduction

Hematophagous insects have evolved special adaptations to ensure a successful bloodmeal from vertebrate hosts, key among which is their saliva. In these bloodfeeders, salivary proteins counteract the pain and itch of the bite, and fight host healing responses such as vasoconstriction and hemostasis [1]. At the same time, salivary compounds can elicit immune responses that are specific to each bloodfeeding species, which in turn can affect the pathogens they transmit [1]. Studies have also shown how vector salivary proteins are useful in disease control, either as markers of biting exposure and risk [2] or as components of vaccines [3-5]. However, few studies have addressed the importance of the post-translational modifications in these proteins.

N-glycosylation is a highly common post- and co-translational modification where the carbohydrate chain is covalently attached to an asparagine residue on a protein containing the consensus sequon Asn-X-Thr/Ser [6]. A vast majority of secreted, non-cytosolic proteins are glycosylated [7]. *N*-glycans have a wide variety of functions, encompassing structural and modulatory properties to the binding of other proteins and cell-cell interactions [8]. As they are secondary gene products, glycoprotein biochemistry varies not only between species but also cell types within the same organism. *N*-glycosylation can affect protein folding, protein stability, ligand binding, and protein antigenicity [6, 9].

In the discipline of glycobiology, insects remain a neglected area of study. Most research has focused on the model fly *Drosophila melanogaster*, where *N*-glycans in particular are important in aspects such as cell adhesion, morphogenesis, and locomotion [10]. As the field of invertebrate glycobiology expands, more work is published on organisms like butterflies, bees and some dipterans [11, 12]; these confirm that insect cells mainly produce oligo- and paucimannosidic *N*-glycans, with some hybrid- and complex-type structures in some species [13]. Compared to *Drosophila*, there has been little research into the *N*-glycans

of bloodfeeders, where the salivary glycoproteins of disease vectors are of special interest considering the role of saliva during hematophagy or vector-host-pathogen interactions.

Tsetse flies are medically and economically important disease vectors in sub-Saharan Africa, where they transmit the parasites that cause human or animal trypanosomiases. Both male and female tsetse flies are obligate blood feeders. When they feed from an infected mammalian host, they ingest bloodstream trypomastigotes, which transform into procyclic trypomastigotes in the midgut [14], and then multiply as epimastigotes in the salivary gland; once there they transform into metacyclic trypomastigotes, which are co-transmitted with saliva during the next blood meal and infect the vertebrate host [15]. Uninfected tsetse flies can inoculate the host with ~4 µg of salivary proteins [16], which can alter host immune responses [17]. However, trypanosome infection induces a severe (~70%) transcriptional downregulation of the tsetse genes that encode for salivary proteins, affecting its anti-hemostatic and anti-clotting properties [18]. In this work we characterize the salivary glycome of the tsetse fly *Glossina* spp., comparing naïve and trypanosome-infected flies. Using highly sensitive liquid chromatography and mass spectrometry, we revealed the presence of several salivary glycoproteins in tsetse saliva, with oligosaccharides composed mainly of pauci-mannose and high-mannose *N*-glycans. Our work presents the first structural analysis of salivary glycans from tsetse flies.

Methods

Ethics statement

Experiments using laboratory rodents were carried out in strict accordance with all mandatory guidelines (European Union directives, including the Revised Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes and the Declaration of Helsinki) and were approved by the ethical committee of the Institute of Tropical Medicine Antwerp, Belgium (PAR011-MC-M-Tryp).

99

100 ## Tsetse fly saliva

101 *Glossina morsitans morsitans* adults were obtained from the tsetse insectary at the Liverpool School of
102 Tropical Medicine (UK). Flies were maintained at 26 ˚C and 65-75% relative humidity and fed for 10 min
103 every two days on sterile, defibrinated horse blood (TCS Biosciences Ltd., Buckingham, UK). Tsetse flies
104 were chilled at 4°C for 20 min, after which the salivary glands were dissected on ice and placed in sterile
105 PBS. The salivary glands were centrifuged at 4 °C, 10,000 rpm for 10 min, and the supernatant collected
106 and stored at -20 °C.

107

108 ## Tsetse infection

109 Teneral (unfed) male *G. m. morsitans* were infected with *Trypanosoma brucei brucei* by combining an
110 aliquot of *T. brucei* (TSW196 strain [19]) infected rat blood with sterile defibrinated horse blood. Flies
111 were bloodfed every two days for 4 weeks until infection of salivary glands was achieved. Saliva was
112 extracted using sterile PBS and stored at -20 ˚C. For evaluation of salivary protein binding to metacyclic
113 trypanosomes, flies were infected with *T. brucei* AnTAR1[16] in defibrinated horse blood supplemented
114 with 10 mM reduced L-glutathion.

115

116 ## Enzymatic deglycosylation for glycoprotein detection

117 Tsetse salivary proteins were treated with peptide-N-glycosidase F (PNGase F, New England Biolabs), which
118 cleaves all *N*-linked glycans except those with an α-1,3 fucose modification of the chitobiose core [20].
119 Deglycosylation was done according to the manufacturer's instructions: briefly, 1x glycoprotein denaturing
120 buffer (5 % SDS, 0.4 M DTT) was added to 10 µg of *G. m. morsitans* salivary proteins and incubated at 100
121 ˚C for 10 min. 1x G7 reaction buffer (0.5 M sodium phosphate pH 7.5), 1% NP40 and 1 µl PNGase F were
122 added and incubated at 37˚C overnight.

Salivary samples were additionally treated with Endoglycosidase H (Endo H; New England Biolabs), which cleaves the chitobiose core of high-mannose and some hybrid *N*-linked glycans [21]. Deglycosylation conditions were the same as described for PNGase F treatment, with an addition of NP40 and the G5 Reaction buffer (50 mM sodium citrate, pH 5.5) used instead of G7; 5000 units of Endo H were used per reaction. Egg albumin (Sigma) was treated in parallel as digestion control for both enzymes.

SDS-PAGE analysis of tsetse salivary proteins

Salivary proteins were resolved on 12% polyacrylamide gels. InstantBlue (Expedeon) and Pierce Glycoprotein Staining kit (ThermoFisher) were used for protein and glycan staining, respectively.

Western Blotting

~2 μg of *G. m. morsitans* salivary proteins were treated with PNGase F (New England Biolabs), resolved by SDS-PAGE and transferred onto a PVDF membrane (GE Healthcare) at 90V for 1 hour. After verification of transfer with Ponceau Red (Sigma-Aldrich), the membrane was blocked for 1 hour (PBS-T [Sigma, US] containing 5% skim milk powder [Sigma]) and incubated in 1:10,000 rabbit-anti-*G. m. morsitans* saliva antibody overnight at 4˚C. Membranes were washed and probed at room temperature for 1 hour with 1:20,000 HRP-labelled goat-anti-rabbit antibody (ThermoFisher). Super Signal West Dura substrate (ThermoFisher) was used for detection.

Concanavalin A (Con A) blotting

~1 μg of tsetse saliva was treated with PNGase F (New England Biolabs). Samples were run on a 12.5% polyacrylamide gel, transferred onto a PVDF membrane, and placed in blocking buffer (1% BSA-PBS-Tw 20 [Sigma]) at 4°C overnight. Following this, the membrane was incubated with 1 μg/ml biotinylated Con A

lectin (Vector Labs, Peterborough) in blocking buffer at room temperature for 1 hour. After washing, the membrane was incubated with 1:100,000 streptavidin-HRP (Vector Labs). SuperSignal West Pico Chemiluminescent substrate (ThermoFisher) was used for detection. Egg albumin (1 µg) was used as a positive control for enzymatic deglycosylation.

Overlay assays with C-type lectins

Saliva samples were treated overnight with PNGase F (New England Biolabs) as described above. Samples were run on a 12.5% polyacrylamide gel, transferred onto a PVDF membrane, and blocked overnight with 1% BSA (Sigma-Aldrich). Membranes were incubated with CTLD4-7Fc (0.5 µg/µl) or DC-SIGN (0.5 µg/µl) (R&D Systems) for 1 hour, washed, and then incubated with anti-human IgG conjugated to HRP for 1 hour. After washing, WestDura substrate (ThermoFisher Scientific) was used to develop the membranes.

Evaluation of salivary protein binding to metacyclic trypanosomes

Metacyclic trypanosomes were isolated from the outflow of infected salivary glands. Parasites were subjected to two subsequent washes with 1.2 mL PBS. Aliquots of those washes were kept for polyacrylamide gel electrophoresis and Western blot analysis. A final pellet of 10^5 parasites was obtained, resuspended in 50 µL PBS and stored at -80°C. As a control, aliquots of 10^5 bloodstream form parasites were prepared following isolation from the blood of infected mice by the mini anion exchange centrifugation technique (mAECT). Parasite washes and lysates were separated on NuPAGE™ 12% Bis-Tris Mini Protein Gels (Invitrogen) at 125 V in an XCell Surelock Mini-Cell (Invitrogen). Gels were used for silverstaining (PageSilver kit, Fermentas) or transfer to Hybond-C nitrocellulose mebranes (GE Healthcare). After overnight blocking in PBS with 5% nonfat dry milk and 0.1% Tween 20, blots were incubated overnight at 4°C with 1 µg/mL purified rabbit polyclonal IgGs (an admix of anti-G. *morsitans* saliva IgG and IgGs against the purified recombinant proteins Tsal1, Tsal2, Tag5, 5'Nucleotidase-related

171 protein and gmmsgp3). As a negative control, blots were incubated with 1 µg/mL purified IgGs from
172 preimmune rabbits. After washing with the blocking solution, membranes were incubated for 1 hour at
173 room temperature with 1:10,000 diluted peroxidase-conjugated goat anti-rabbit IgG (Sigma, A6154).
174 After 4 washes in PBS with 0.1% Tween 20, blots were developed with Immobilon chemiluminescent
175 horseradish peroxidase substrate (Millipore) and exposed to an autoradiography film (GE Healthcare).
176

177 ## Mass spectrometry analysis of salivary proteins

178 To identify the glycoproteins that were susceptible to PNGase F cleavage, 10 µg of salivary proteins were
179 resolved in a 12 % polyacrylamide gel and Coomassie stained. Bands of interest were sent to the Dundee
180 University Fingerprints Proteomics Facility, where they were subjected to in-gel trypsination and then
181 alkylated with iodoacetamide. Peptides were analyzed by liquid chromatography-tandem mass
182 spectrometry (LC-MS/MS) in a Thermo LTQ XL Linear Trap with a nano-LC.
183

184 The data was supplied in MASCOT format. The gi numbers for the top hits in each band were searched in
185 NCBI Protein (http://www.ncbi.nlm.nih.gov/protein) to yield the FASTA format of the protein sequence.
186 This was then queried in PROWL (http://prowl.rockefeller.edu/) to reveal the predicted molecular weight
187 and also to predict tryptic peptides in the sequence. The FASTA protein sequence was also queried in the
188 SignalP 4.0 Server software[187] to predict the signal peptide location and NetNGlyc 1.0
189 (http://www.cbs.dtu.dk/services/NetNGlyc/) to reveal potential *N*-glycosylation sites.
190

191 ## Release of tsetse salivary *N*-linked glycans for structural analysis

192 *N*-linked glycans were released by in-gel deglycosylation using PNGase F (QA Bio) as described by Royle *et*
193 *al*. [22]; in addition, tsetse saliva was treated with peptide-N-glycosidase A (PNGase A, New England
194 Biolabs), which releases all *N*-linked glycans, including those with an α-1,3 fucose modification of the

chitobiose core [20]. Briefly, peptides were released from gel pieces by overnight incubation at 37°C with trypsin in 25 mM ammonium bicarbonate. The supernatant was dried, re-suspended in water and heated at 100°C for 10 min to deactivate the trypsin. After drying by vacuum centrifugation, the tryptic peptide mixture was incubated with PNGase A in 100 mM citrate/phosphate buffer (pH 5) for 16 hours at 37°C [23]. PNGase F and PNGase A released *N*-glycans were separated from protein and salts by LudgerClean™ Protein Binding Plate (Ludger Ltd., Oxfordshire, UK). Glycans pass straight through the protein binding membrane. Wells are flushed with extra water to ensure full recovery, and then dried by vacuum centrifugation prior to fluorescent labelling.

Fluorescent labelling (2-aminobenzamide or procainamide) and purification

Released *N*-glycans were fluorescently labelled via reductive amination by reaction with 2-aminobenzamide (2-AB) using a Ludger 2-AB Glycan Labelling Kit (Ludger Ltd., Oxfordshire, UK), or a Ludger Procainamide Glycan Labelling Kit (Ludger Ltd., Oxfordshire, UK), both containing 2-picoline borane. Released glycans were incubated with labelling reagents for 1 hour at 65°C [24]. 2-AB labelled glycans were cleaned up using LudgerClean T1 Cartridges (Ludger Ltd., Oxfordshire, UK). Procainamide labelled glycans were cleaned up using LudgerClean S cartridges (Ludger Ltd., Oxfordshire, UK). In both cases, fluorescently labelled glycans were eluted with 1 mL water. Samples were evaporated and then re-suspended in water for further analysis.

Exoglycosidase sequencing

Exoglycosidase digestion was performed according to Royle et al. [22]. The released, 2-AB labelled *N*-glycans were incubated with exoglycosidases at standard concentrations in a final volume of 10 µL in 50 mM sodium acetate (for incubations with JBAM, 250 mM sodium phosphate, pH 5.0 was used) for 16 hours at 37°C. Glycans were incubated with different exoglycosidases in different sequences: (i)

Streptococcus pneumonia β-N-acetylglucosaminidase (GUH, New England Biolabs); (ii) Jack bean α-(1-2,3,6)-Mannosidase (JBAM, QA-Bio); (iii) Bovine kidney α-(1-2,3,4,6)-Fucosidase (bkF; Sigma-Aldrich). After digestion, samples were separated from the exoglycosidases by binding onto a LudgerClean Post-Exoglycosidase clean-up plate (Ludger Ltd., Oxfordshire, UK) for 60 min followed by elution of the glycans from the plate with water.

UHPLC analysis

2-AB labelled samples were analyzed by HILIC-UPLC using an ACQUITY UPLC® BEH-Glycan column (1.7 µm, 2.1 x 150 mm) at 60 °C on a Dionex UltiMate 3000 UHPLC instrument (Thermo, UK) with a fluorescence detector (λex = 250 nm, λem = 428 nm), controlled by Chromeleon data software version 6.8. Gradient conditions were: 0 to 53.5 min, 24% A (0.4 mL/min); 53.5 to 55.5 min, 24 to 49 % A (0.4 mL/min); 55.5 to 57.5 min, 49 to 60% A (0.4 to 0.25 mL/min); 57.5 to 59.5 min, 60 % A (0.25 mL/min); 59.5 to 65.5 min, 60 to 24% A (0.4 mL/min); 65.5 to 66.5 min, 24% A (0.25 to 0.4 mL/min); 66.5 to 70 min 24% A (0.4 mL/min). Solvent A was 50 mM ammonium formate; solvent B was acetonitrile (Acetonitrile 190 far UV/gradient quality; Romil #H049, Charlton Scientific). Samples were injected in 24% aqueous/76% acetonitrile with an injection volume of 25 µL. Chromeleon software retention index function with a cubic spline fit was used to allocate GU values to peaks. 2-AB labelled glucose homopolymer (Ludger Ltd., Oxfordshire, UK) was used as a system suitability standard as well as an external calibration standard for GU allocation on the system.

Online Hydrophilic Interaction Liquid Chromatography-Solid Phase Extraction LC-MS analysis

Procainamide labelled glycans were prepared in 0.1 % TFA (v/v) in 78% acetonitrile (v/v) and desalted on-line using hydrophilic interaction liquid chromatography solid phase extraction prior to direct elution into the mass spectrometer. Samples were applied onto a HILIC trapping column (MilliporeSigma

1.50484.0001 SeQuant® HPLC Guard Column with ZIC-HILIC [5 µm] Sorbent Packing Media, 5 x 0.3mm) at a flow rate of 1.5 µl/min using an UltiMate 3000 LC (Thermo Scientific, Massachusetts). The trap was washed for 4 min with 0.1% formic acid in 90% ACN (v/v) followed by elution of procainamide labelled glycans using an isocratic gradient of 0.1 % formic acid in 27 % ACN (v/v) for 12 min. Glycans were analysed by electrospray ionization tandem mass spectrometry (ESI-MS) on an amaZon speed ETD ion trap MS (Bruker, Massachusetts) in positive ion mode with a nitrogen flow of 10 L/min and capillary voltage of 4500 V.

LC-ESI-MS and LC-ESI-MS/MS analysis

Procainamide labelled samples were analyzed by HILIC-UHPLC-ESI-MS with fluorescence detection (λ_{ex} = 310 nm, λ_{em} = 370 nm), using the same UHPLC conditions as detailed above for 2-AB analysis, with the exception of column temperature (set to 40 °C). ESI-MS and MS/MS detection were carried out using an amaZon speed ETD ion trap MS (Bruker, Massachusetts) as above, with the top three precursors ions selected for MS/MS fragmentation.

Results

Identification of tsetse salivary glycoproteins

Bioinformatic analysis was performed to identify potential glycosylation sites on tsetse salivary proteins, looking at proteins having the Asn-X-Ser/Thr sequons. The NetNGlyc server prediction tool [25] identified that 72% of *Glossina* proteins have at least one potential glycosylation site (Supplementary Table 1). However, although the consensus sequence is a prerequisite for the addition of *N*-glycans to the asparagine, it does not guarantee their glycosylation status *in vivo*. The presence of glycoproteins was then confirmed by Schiff's staining of SDS-PAGE separated proteins, which indicated several glycoproteins

267 migrating with different apparent molecular masses (Figure S1). Tsetse salivary proteins contained in the
268 migrating bands were identified by mass spectrometry (Supplementary Tables 2 and 3) and
269 complemented the assignment using published data [17, 18, 26].
270
271 To identify the type of glycosylation (*N*- or *O*-linked) present in tsetse salivary glycoproteins, we first
272 released the *N*-glycans and analyzed the deglycosylated proteins by SDS-PAGE fractionation and mass
273 spectrometry. Treatment with PNGase F revealed several proteins that showed an electrophoretic shift
274 after deglycosylation (Figure S2). Furthermore, PNGase F treatment allowed the fractionation and
275 visualization of salivary proteins with an apparent high molecular mass (Figure S2, lane 2). Proteomic
276 analysis revealed 4 bands with notable shift in electrophoretic migration: 5' Nucleotidase-related protein
277 (5'Nuc), TSGF 2/Adenosine deaminase, TSGF 1, and Tsal 1/2. No proteins were detected by Schiff's
278 staining (Figure S1) after treatment, suggesting that the main type of sugars linked to tsetse salivary
279 glycoproteins are *N*-glycans.
280
281 To better characterize the type of *N*-glycosylation present, salivary glycoproteins were treated with Endo
282 H (Supplementary Figure S3), which cleaves *N*-glycans with at least 3 mannose residues where the α1,6
283 mannose branch is attached to another mannose [27]. These results showed an SDS-PAGE band
284 migration similar to that observed following PNGase F treatment (Figure S2). Mass spectrometric protein
285 identification of the main migrating bands also detected similar glycoproteins, and the protein
286 identifications, number of potential glycosylation sites, and peptide coverage is shown in Supplementary
287 Table 3. Taken together, these results indicate the presence of few key glycoproteins in tsetse fly saliva,
288 and more importantly, the presence of several high mannose or hybrid type *N*-glycans.
289
290 Structural characterization of *G. morsitans* salivary *N*-glycans

For full structural characterisation of the *N*-glycome of *G. m. morsitans* saliva, glycans where released by PNGase F digestion, purified and tagged with 2-AB, a fluorescent label for chromatographic detection. HILIC-UHPLC analysis revealed 13 peaks that correspond to a variety of potential high mannose and hybrid *N*-glycan structures (Figure 1A), in line with the results of the Endo H digest. The peak of highest intensity (abundance) corresponds to the core structure $Man_3GlcNAc_2$-2AB. After treatment with PNGase A, which cleaves all types of *N*-glycans (including those with core fucose residues in an α-1,3 linkage to the reducing end GlcNAc), the profile of oligosaccharides did not show any difference to the one obtained by PNGase F digestion (Supplementary Figure S4). This suggests the absence of α-1,3 core fucosylated structures in *G. m. morsitans* saliva, and indicates that the single core fucosylated structure identified (peak 3, Figure 1A) carries an α-1,6 linked core fucose.

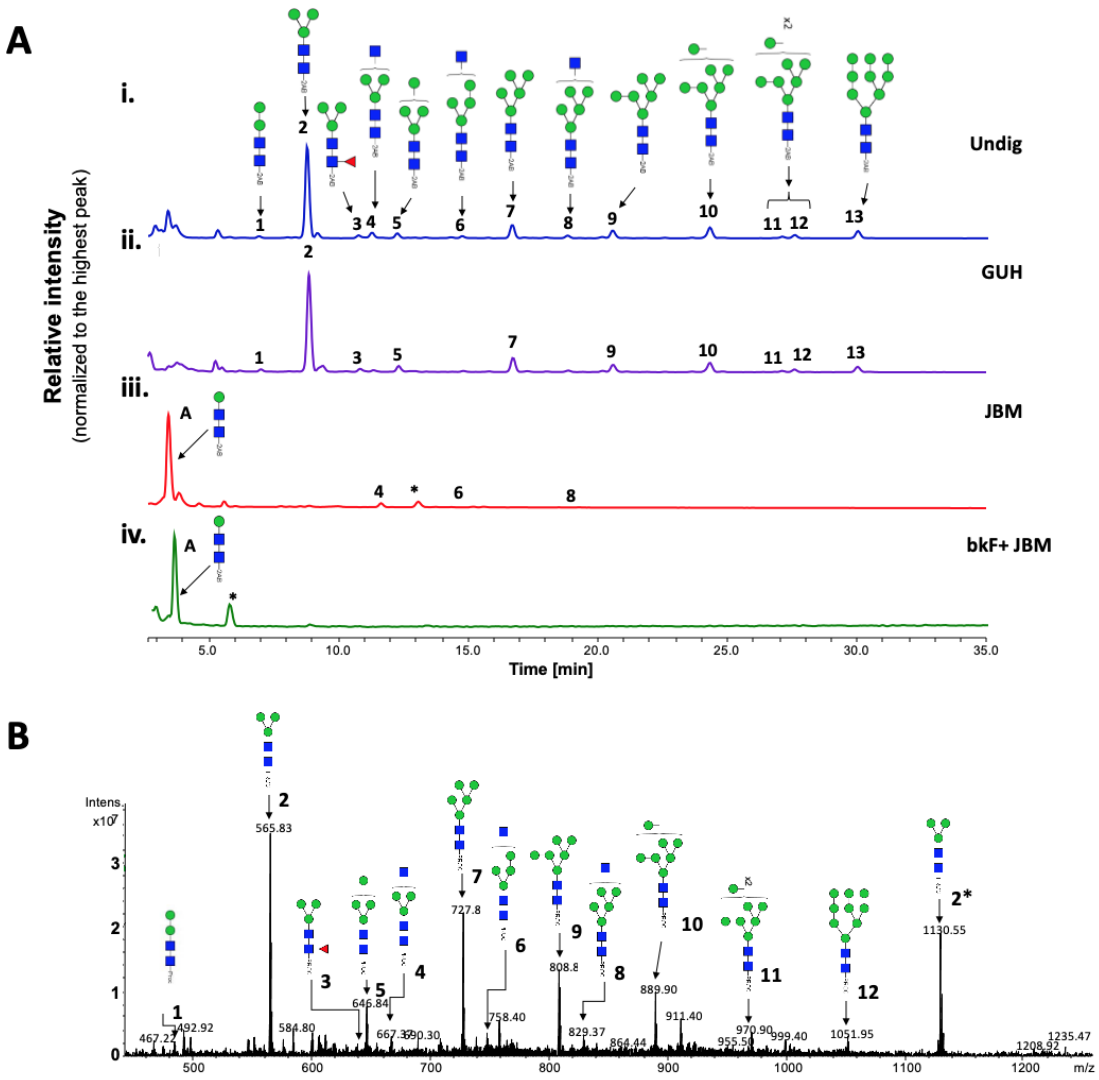

Figure 1. Tsetse fly salivary glycoproteins are composed mainly of paucimannose and oligomannose glycans. [A] Profile of salivary *N*-glycans from teneral (young, unfed) flies, before and after digestion with exoglycosidases. Aliquots of the total PNGase F-released 2-AB-labeled *N*-glycan pool were either undigested (i) or incubated with a range of exoglycosidases (ii-iv). (i) Undig, before digestion; (ii) GUH, *Streptococcus pneumonia* in *E. coli* β-N-acetylglucosaminidase; (iii) JBM, Jack bean α-Mannosidase; (iv) bkF, Bovine kidney α-fucosidase. Following digestion, the products were analysed by HILIC-(U)HPLC. Peaks labelled A correspond to the product of complete digestion with JBM; those labelled with an asterisk refer to buffer contaminants. The percent areas and structures of the different glycans are listed in Table 1. [B] Positive-ion ESI-MS spectrum of procainamide-labelled *N*-glycans from teneral tsetse fly saliva. Numbers refer to the structures shown in Table 1. Asterisk (*) refers to *m/z* 1130.55 as $[M+2H]^{2+}$ ion. Green circle, mannose; blue square, *N*-Acetylglucosamine; red triangle, fucose; Proc, procainamide.

313 To further characterise the structure of these glycans, samples were treated with exoglycosidases of
314 different specificities to confirm monosaccharide linkages: β-*N*-Acetylglucosaminidase (GUH; Figure 1A -
315 ii), Jack Bean α-(1-2,3,6)-Mannosidase (JBM; Figure 1A - iii), bovine kidney fucosidase (bkF; Figure 1A - iv).
316 GUH resulted in a reduction of peaks 4, 6 and 8, indicating the presence of a terminal non-reducing β-
317 GlcNAc in these structures. This was further corroborated after JBM digestion, where most glycans (with
318 the exception of peaks 4, 6 and 8, see Table 1) were completely digested as shown by the appearance of
319 a new peak identified as βMan-βGlcNAc-βGlcNAc-2AB (Figure 1A - iii). Finally, sequential treatment with
320 bkF followed by JBM resulted in the loss of all peaks in the chromatogram. Peak information after
321 enzymatic treatment is further detailed in Table 1.
322
323 For secondary confirmation of the salivary *N*-glycan structures by mass spectrometry, released
324 oligosaccharides were labelled with procainamide and then analyzed by positive-ion ESI-MS and ESI-
325 MS/MS. The resulting mass spectra confirms the findings by HILIC-UHPLC analysis, showing the presence
326 of 12 $[M+H]^{2+}$ ions with *m/z* 565.74, 646.74, 727.79, 808.81, 889.84, 970.87, 1051.90 (corresponding to
327 $Man_{3\text{-}9}GlcNAc_2$-Proc), in addition to three complex type glycans with truncated antenna; i.e.
328 $Man_3GlcNAc_2Fuc$-Proc, $Man_3GlcNAc_3$-Proc, and $Man_4GlcNAc_3$-Proc (Figure 1B; Supplementary Table 4).
329 Structural topology was confirmed by positive ion ESI-MS/MS fragmentation spectra, including the most
330 abundant species $Man_3GlcNAc_2$-Proc ($[m/z]^+$ 1130.49) as well as $Man_3GlcNAc_2Fuc$-Proc ($[m/z]^+$ 1276.57)
331 and $Man_3GlcNAc_3$-Proc ($[m/z]^+$ 1333.59) (Figure S5; Supplementary Table 5). Details of all *N*-glycans and
332 the MS/MS diagnostic ions used in their identification can be found in Supplementary Table 4. Overall,
333 these results suggest that tsetse salivary glycans consist mainly of highly processed $Man_3GlcNAc_2$ in
334 addition to several other paucimannose, oligomannose, and few hybrid-type glycans.
335
336 **Glycosylation profile of tsetse saliva remains unaffected during a trypanosome infection**

337 Since *T. brucei* infection affects the composition of tsetse saliva [18], we investigated if infection changes
338 salivary glycosylation as well. Initially, we compared the salivary profiles of uninfected flies with those that
339 had either a salivary gland or a midgut infection with *T. brucei* by SDS-PAGE (Supplementary Figure S6).
340 When samples were normalized by protein concentration, there were no major changes in the profile of
341 salivary proteins in the different physiological states.
342

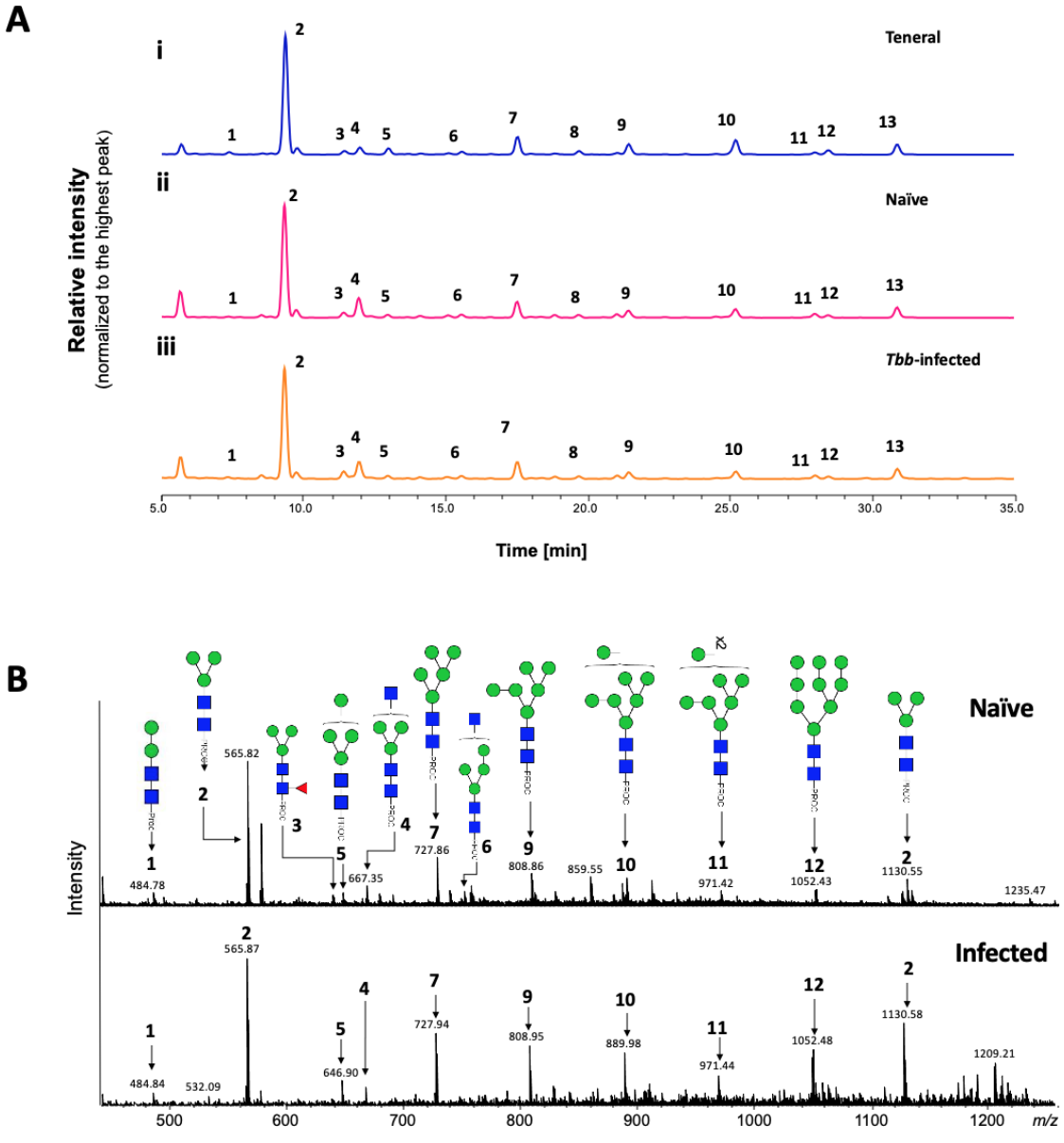

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Figure 2. Analysis of tsetse salivary *N*-linked glycans in teneral, naïve and trypanosome-infected flies. [A] Comparison of HILIC-(U)HPLC profiles of salivary *N*-glycans released by PNGase F. Analysis of 2AB-labelled glycans from (i) teneral, (ii) naïve, and (iii) trypanosome-infected saliva. Relative abundances are indicated in table 2. Tbb, *Trypanosoma brucei brucei*. [B] Positive-ion ESI-MS analysis of procainamide labelled *N*-glycans from adult naïve and trypanosome-infected saliva. Spectra are shown for naïve (top) and trypanosome-infected (bottom) saliva. Numbers refer to the structures shown in Table 1. Green circle, mannose; blue square, *N*-Acetylglucosamine; red triangle, fucose; Proc, procainamide.

We then determined whether *T. b. brucei* infection alters the structure of salivary *N*-glycans. Oligosaccharides were released with PNGase F, labelled with procainamide, and then analyzed by HILIC coupled with ESI-MS. Figure 2A shows the HILIC chromatogram, where no change is observed in the saliva *N*-glycan population following infection of the salivary gland. A comparison of the relative percentage areas of glycan peaks identified in teneral, naïve and infected fly saliva (Table 2) showed no quantitative differences after infection either. This is confirmed by the MS spectra (Figure 2B) which shows that salivary glycan structures and abundances remain unaffected by infection with *T. brucei*. However, when comparing teneral versus naïve (bloodfed) flies or infected flies, there seemed to be a slight variation in the abundance of some structures (Table 2: peaks 4, 9, 10, 13), potentially an effect of blood ingestion.

Trypanosoma infection does not alter immune reactivity of tsetse salivary glycoproteins with antibodies

Next, we investigated whether a trypanosome infection alters the immune reactivity of tsetse salivary glycoproteins. By immunoblotting, we compared the saliva of flies with midgut or salivary gland infection, before and after treatment with PNGase F (Figure 3). After probing with a polyclonal anti-*G. morsitans* saliva rabbit serum, recognition of control *G. morsitans* saliva before and after cleavage of the glycans appears unaffected. However, during salivary gland infection the polyclonal serum only detected the high molecular weight proteins (100 kDa-130 kDa) after glycans were cleaved. The effect is more readily seen here possibly due to the downregulation of other salivary proteins during infection, and seems to be

concealed both in the saliva of naïve flies and those with midgut infection. Interestingly, saliva from trypanosome-infected salivary glands displayed an antigenic ~20 kDa band that is faintly seen by SDS-PAGE (Supplementary Figure S6), and is absent from uninfected saliva following Western blotting. We suggest these probably represent proteolytic products of salivary proteins that are formed as a result of the trypanosome infection in the gland [28].

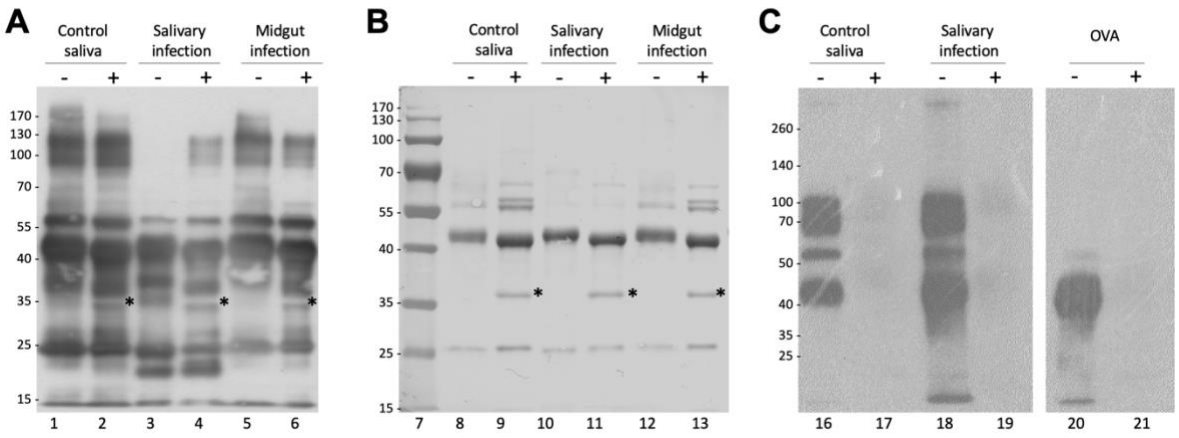
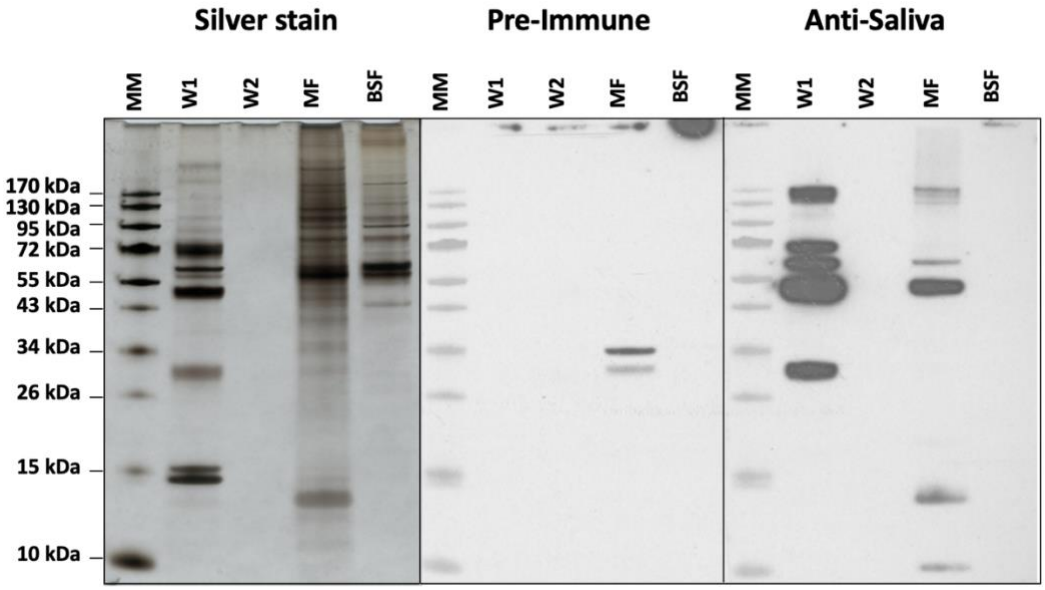


Figure 3. Analysis of the effects of infection on immunogenicity of tsetse fly saliva. (A) 2 µg of *G. m. morsitans* salivary proteins were treated (+) or untreated (-) with PNGase F, fractionated by SDS-PAGE, transferred onto a PVDF membrane, and probed with an anti-*G. m. morsitans* saliva antibody. **(B)** Uniform protein loading for Western blot was confirmed by nigrosine staining of proteins transferred to PVDF membrane. **(C)** Con A blotting analysis of tsetse salivary glycoproteins from naïve and trypanosome-infected flies. OVA, egg albumin positive control. Asterisk indicates PNGase F enzyme band.

Glycosylated salivary proteins bind onto metacyclic trypanosomes

Binding of saliva proteins to metacyclic trypanosomes was evaluated by isolating and washing metacyclics from *T. brucei*-infected *G. morsitans* salivary glands. Presence of saliva proteins in the washes and on metacyclic trypanosomes was revealed by Western blot using rabbit anti-*G. morsitans* saliva IgGs (Figure 4). Specificity of salivary protein detection was assured by including rabbit pre-immune IgGs and a lysate of an equal number of bloodstream trypanosomes as controls. The second parasite wash was devoid of

388 detectable levels of saliva proteins, whereas the metacyclic parasite lysates contained various protein
389 bands that were specifically recognized by the immune serum (Figure 4). Based on their apparent
390 molecular masses on SDS-PAGE and mass spectrometry identification (Figure S2 and Figure S1), these
391 components likely correspond to 5'Nucleotidase-related protein sgp3, TSGF and the Tsal glycoproteins.
392 Two additional unidentified < 15 kDa bands were specifically detected using the anti-saliva IgGs.
393 Interestingly, the abundant, non-glycosylated TAg5 protein in saliva was found not to bind to the
394 metacylic trypanosome surface.
395



396

397 **Figure 4. Analysis of the binding of salivary proteins to metacyclic trypanosomes.** Silver stained protein profiles and Western
398 blot analysis to detect the presence of salivary proteins on tsetse salivary gland-derived trypanosomes. Two subsequent washes
399 (W1 and W2) of metacyclic parasites (MF, equivalent of 3×10^4 parasites loaded on gel) isolated from infected tsetse fly salivary
400 glands and a corresponding sample of trypanosomes purified from mouse blood (BSF, equivalent of 3×10^4 parasites loaded).
401 Proteins bands were revealed with purified rabbit anti-*G. morsitans* saliva IgGs and pre-immune IgGs as a control, and
402 development with peroxidase-coupled goat anti-rabbit IgG. Asterisk indicates salivary proteins in the metacyclic trypanosome
403 lysate.

404

405 ***N*-glycans from *G. morsitans* salivary glycoproteins are recognised by the mannose receptor and DC-SIGN**

406 To further understand the biological role of the *G. morsitans* salivary *N*-glycans, we explored their
407 potential recognition by cells from the immune system. Endocytic c-type lectin receptors, such as
408 macrophage mannose receptor (CTLD) and the dendritic cell-specific ICAM3 grabbing nonintegrin (DC-
409 SIGN), can recognize exposed mannose residues on glycoproteins. Using recombinant CTLD4-7-Fc and
410 recombinant Human DC-SIGN Fc Chimera proteins, the carbohydrate-binding domains from these two
411 receptors, we performed overlay assays using saliva before and after treatment with PNGase F (Figure 5).
412 Our results showed that CTLD4-7-Fc recognized at least 4 glycoprotein bands migrating around 45-75 kDa
413 in *G. morsitans* saliva, while DC-SIGN recognized only two of them around 70 kDa. Recognition of these
414 bands by either lectin disappeared after PNGase F treatment, confirming specificity of binding to *N*-linked
415 mannosylated glycans. However, the overall recognition of tsetse salivary glycoproteins by either lectin is
416 much lower compared to that observed with Con A (Figure 3, lane 16).
417

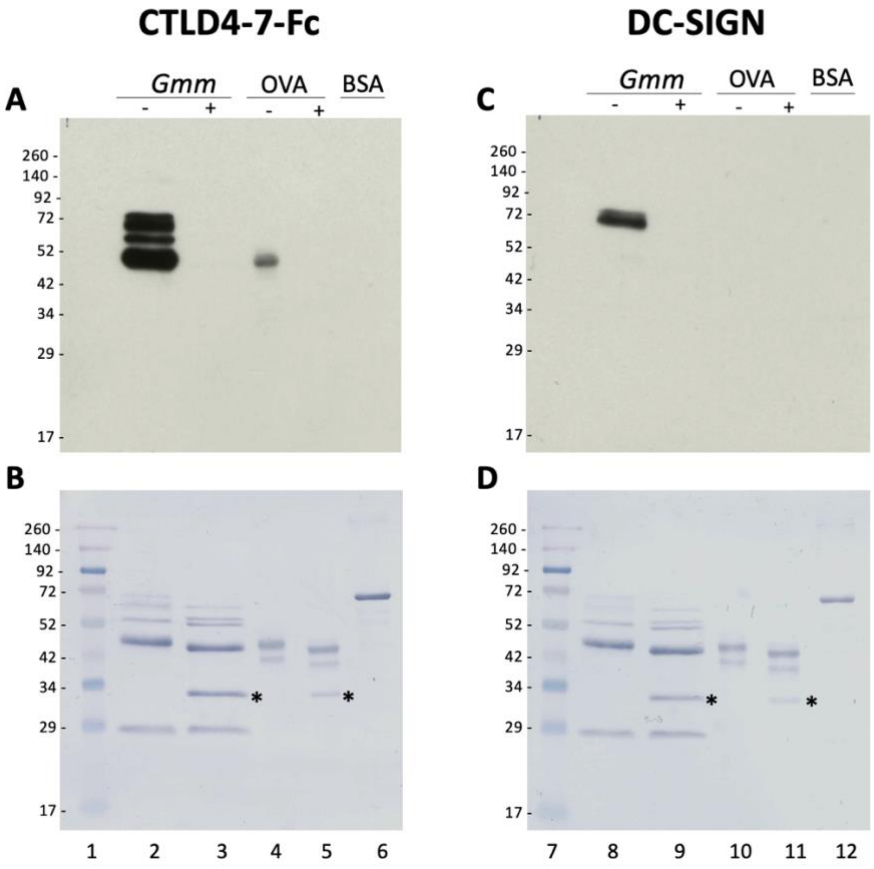


Figure 5. Tsetse salivary glycans are recognized by C-type lectins Mannose Receptor and DC-SIGN. 2 µg of *Glossina morsitans* saliva (*Gmm*) were untreated (-) or treated (+) with PNGase F and then processed for overlay assays using either recombinant CTLD4-7-Fc (A) or DC-SIGN (B). MWM, lanes 1 and 7; *Gmm* saliva, lanes 2, 3, 8 and 9; OVA, egg albumin positive control (lanes 4, 5, 10 and 11); BSA, bovine serum albumin negative control (lanes 6 and 12). Nigrosine-stained membranes (B, D) are shown as loading controls for (A) and (B), respectively. Asterisk indicates PNGase F enzyme.

Discussion

We reveal for the first time the composition and structure of the oligosaccharides modifying the salivary proteins of the tsetse fly *G. m. morsitans*, vector of African trypanosomiasis. Through enzymatic analysis coupled with highly sensitive LC and ESI-MS/MS, we found that salivary *N*-glycans in *G. morsitans* saliva are mainly represented by the paucimannose $Man_3GlcNAc_2$ structure, with the addition of a few hybrid-

type oligosaccharides. Three hybrid structures with terminal GlcNAc residues were also detected: $Man_3GlcNAc_3$, $Man_4GlcNAc_3$ and $Man_5GlcNAc_3$. In addition, we only found one fucosylated structure ($FucMan_3GlcNAc_2$), indicating that most of the sugars do not undergo further processing after trimming of the mannose residues.

Research on insect glycans shows these are mostly oligomannose, although complex structures with terminal GlcNAc are often found in some species [12]. Core fucosylation (both α1-3 and α1-6) is quite common in invertebrate glycans [33], and can cause allergic reactions in vertebrates [34]. Overall, tsetse salivary glycans fit these observations. Some studies have suggested *G. m. centralis* saliva contained as many as seven glycoproteins [35], while *G. m. morsitans* contained four salivary glycoprotein bands [26], and predicted *N*-glycosylation sites in TSGF 1 and 2 [36], and Gmmsgp 2 and 3 [16]. Caljon *et al.* (2010) reported on the putative glycosylation of salivary 5'nucleotidase-related apyrase (5'Nuc), a salivary protein that interrupts formation of the hemostatic plug by hydrolyzing ATP and ADP [37]. NetNGlyc identifies four glycosylation sites in the peptide sequence, and Caljon et al report a ~5kDa loss in mass after PNGase F treatment (in agreement with our results). Assays with recombinant non-glycosylated form of 5'Nuc suggest sugars are not essential for its activity, but they might be important for secretion and solubility. However, the role of these glycan modifications in the tsetse salivary proteins remains to be elucidated.

The presence of glycoproteins has also been described in the salivary glands of *An. gambiae* (some being female-specific) [52], *An. stephensi* [53], *Ae. albopictus* [54], and *Phlebotomus duboscqi* sandflies [55]. However, the most complete structural characterization of salivary glycoproteins in disease vectors to date has been that of *Lutzomyia longipalpis*, vector of visceral leishmaniasis in the Americas [29]. This sand fly species makes mostly oligomannose *N*-glycans, with $Man_5GlcNAc_2$ being the most abundant

structure. When we compare the salivary *N*-glycomes of *G. morsitans* with that of the sand fly *Lu. longipalpis*, it is possible to observe that both profiles are strikingly similar regarding the high content of mannosylated species, except that in *Glossina* the major glycan is the tri-antennary core $Man_3GlcNAc_2$ structure, compared to the dominant $Man_5GlcNAc_2$ in sandflies. In general, the dominance of mannosylated *N*-glycans that was found in both species suggests a conserved protein glycosylation pathway among hematophagous dipterans; in addition to potentially modulate pathogen transmission, this raises the question on the functional role(s) these mannosylated glycans may have during insect blood feeding.

The abundance of paucimannose and oligomannose sugars in tsetse salivary gycoproteins leads us to hypothesize about how these might interact with the host immune system. Several cells of the dermal immunological repertoire harbor receptors with carbohydrate binding domains [45], such as the mannose receptor (MR), which is expressed in populations of macrophages and dendritic cells, and participates in antigen presentation and the clearance of molecules [46]. The C-type lectin-like domain (CTLD) of the MR binds glycosylated molecules with terminal Man, Fuc or GlcNAc; our work shows that a recombinant CTLD4 can recognize several *N*-glycans from tsetse saliva. Another example of these receptors is the DC-SIGN, a dendritic cell receptor involved in antigen presentation and the initial detection of pathogens. Its carbohydrate recognition domains bind to high-mannose oligosaccharides, which mediate dendritic cell recognition of pathogens like *Mycobacterium tuberculosis* and *Leishmania* [47, 48]. We also show here that a recombinant fraction of the DC-SIGN recognizes some salivary glycoproteins from tsetse saliva. Notably, the DC-SIGN seems to recognize fewer tsetse glycoproteins than the mannose receptor, possibly due to its specificity for high-mannose glycan structures [49], which would not recognize the most abundant $Man_3GlcNAc$ glycan species.

Salivary glycans might also be interacting with the lectin complement system, which is initiated when the mannan-binding lectin or ficolins bind to carbohydrates on pathogens. Mannan-binding lectin binds to several sugars, including Man, GlcNAc and Fuc, through which it recognizes pathogens like *T. cruzi*, *Leishmania* and *Plasmodium* [50]. Ficolins on the other hand bind to glycans containing disulfated *N*-acetylactosamine, terminal Gal or GlcNAc [51]. Although there are no reports of this pathway activation by either *Glossina* or *Trypanosoma*, the potential masking of metacyclics with salivary glycoproteins could be a mechanism to reduce destruction by the lectin pathway of complement or activation of DC-SIGN-dependent immune response.

Interestingly, a tsetse salivary gland infection with *T. brucei* did not alter the glycosylation profile in saliva samples, suggesting that it does not seem to affect the biosynthesis or transfer of *N*-glycans to proteins in salivary epithelial cells. This was a surprising finding given that trypanosomes causes such a profound transcriptional downregulation of most tsetse salivary proteins [16]. Furthermore, when we investigated the immune reactivity of salivary proteins with antibodies using an anti-*G. morsitans* polyclonal serum, a larger number of antigenic epitopes were detected in high molecular weight proteins only after PNGase F deglycosylation (more evident in trypanosome-infected samples). It is possible that some salivary epitopes are being masked from the immune system by glycans. Even though parasite infection does not affect salivary glycosylation, we noted slight variations in the relative abundance of some glycan structures in unfed versus bloodfed flies; this could be due to the bloodmeal itself causing changes in the salivary protein glycosylation, an effect that would be interesting to explore further.

In this work we also evaluated the binding of salivary proteins to the metacyclic trypanosomes (which infect the vertebrate host). We unexpectedly found that that only glycosylated salivary proteins (e.g. 5'Nucleotidase related protein sgp3 and TSGF and Tsal) associate with the trypanosome surface. The

biological significance of this finding remains to be determined, but it may enable immunomodulatory activity in the immediate parasite microenvironment during the early infection processes in the skin [38]. In some cases, a vector's salivary proteins may associate to the surface of a pathogen, and affect how the vertebrate host's immune system recognises and eliminates the invader. An example of this is the Salp15 salivary glycoprotein of *Ixodes* ticks, which binds to the outer surface protein C of *Borrelia burgdorferi*, creating a protective coat against complement-mediated killing [39-41]. There also evidence that some *Aedes* salivary proteins may interact with dengue virions to favour their transmission [42, 43]. MosGILT, a protein from the salivary glands of *Anopheles*, also binds to the surface of *Plasmodium* sporozoites; however, in this case it negatively affects the traversal activity of parasites and reduces their ability to infect the host [44].

We did not detect *O*-glycans by either HILIC or MS (data not shown), which coincides with our recent findings in *Lutzomyia longipalpis* saliva [29]. This indicates that either these sugars are made in very low abundance by salivary gland cells of these insects, or they may be structurally different compared to mammalian *O*-glycans. Nevertheless, the *G. morsitans* sialome describes the presence of at least nine mucin polypeptides, which are members of the hemomucin family [17], and predictions showed that these proteins have anywhere between 12 and 40 putative *O*-linked glycosylation sites. In addition, *Glossina* species express a large family of peritrophins and peritrophin-like glycoproteins in the peritrophic matrix [30, 31], which protects the fly from harmful components present in the bloodmeal. Tsetse peritrophins contain one or more mucin domains that are likely modified with *O*-linked glycosaminoglycans (GAGs) [32]. It remains to be seen whether insect salivary glycoproteins, containing mucin domains, are modified by GAGs or completely lack canonical (GalNAc-linked) mammalian *O*-glycosylation.

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Author's contributions

Designed experiments (RPK, KMS, CW, CG, JDBA, AA-S), performed experiments (RPK, KMS, CW, CR, SP, KWK, RG, GC) analysed the data (RPK, KMS, CW, CR, SP, KWK, RG, GC, JVDA, DS, MJL, AA-S), wrote the manuscript (RPK, KMS, CW, AA-S). All authors reviewed and approved the manuscript.

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Availability of Data and Materials

All data generated or analysed during this study are included in this published article (and its supplementary information files).

Ethics approval and consent to participate

Not applicable

Competing interests

The author(s) declare(s) that they have no competing interests.

References

1. Gillespie RD, Mbow ML, Titus RG: **The immunomodulatory factors of bloodfeeding arthropod saliva**. *Parasite Immunol* 2000, **22**(7):319-331.
2. Abdeladhim M, Kamhawi S, Valenzuela JG: **What's behind a sand fly bite? The profound effect of sand fly saliva on host hemostasis, inflammation and immunity**. *Infect Genet Evol* 2014, **28**:691-703.
3. Giunchetti RC, Correa-Oliveira R, Martins-Filho OA, Teixeira-Carvalho A, Roatt BM, de Oliveira Aguiar-Soares RD, Coura-Vital W, de Abreu RT, Malaquias LC, Gontijo NF *et al*: **A killed *Leishmania* vaccine with sand fly saliva extract and saponin adjuvant displays immunogenicity in dogs**. *Vaccine* 2008, **26**(5):623-638.
4. Valenzuela JG, Belkaid Y, Garfield MK, Mendez S, Kamhawi S, Rowton ED, Sacks DL, Ribeiro JM: **Toward a defined anti-*Leishmania* vaccine targeting vector antigens: characterization of a protective salivary protein**. *J Exp Med* 2001, **194**(3):331-342.
5. Pandey RK, Dahiya S, Mahita J, Sowdhamini R, Prajapati VK: **Vaccination and immunization strategies to design *Aedes aegypti* salivary protein based subunit vaccine tackling Flavivirus infection**. *Int J Biol Macromol* 2019, **122**:1203-1211.
6. Imperiali B, O'Connor SE: **Effect of N-linked glycosylation on glycopeptide and glycoprotein structure**. *Curr Opin Chem Biol* 1999, **3**(6):643-649.
7. Wyss DF, Wagner G: **The structural role of sugars in glycoproteins**. *Curr Opin Biotechnol* 1996, **7**(4):409-416.
8. Varki A, Lowe JB: **Biological Roles of Glycans**. In: *Essentials of Glycobiology.* Edited by nd, Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME. Cold Spring Harbor (NY); 2009.
9. Stanley P, Taniguchi N, Aebi M: **N-Glycans**. In: *Essentials of Glycobiology.* Edited by rd, Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Darvill AG, Kinoshita T, Packer NH *et al*. Cold Spring Harbor (NY); 2015: 99-111.
10. Katoh T, Tiemeyer M: **The N's and O's of *Drosophila* glycoprotein glycobiology**. *Glycoconj J* 2013, **30**(1):57-66.
11. Walski T, De Schutter K, Van Damme EJM, Smagghe G: **Diversity and functions of protein glycosylation in insects**. *Insect Biochem Mol Biol* 2017, **83**:21-34.
12. Vandenborre G, Smagghe G, Ghesquiere B, Menschaert G, Nagender Rao R, Gevaert K, Van Damme EJ: **Diversity in protein glycosylation among insect species**. *PLoS One* 2011, **6**(2):e16682.
13. Shi X, Jarvis D: **Protein N-Glycosylation in the Baculovirus-Insect Cell System**. *Curr Drug Targets* 2007, **8**(10):1116–1125.
14. Rose C, Casas-Sanchez A, Dyer NA, Solorzano C, Beckett AJ, Middlehurst B, Marcello M, Haines LR, Lisack J, Engstler M *et al*: ***Trypanosoma brucei* colonizes the tsetse gut via an immature peritrophic matrix in the proventriculus**. *Nat Microbiol* 2020. doi:10.1038/s41564-020-0707-z
15. Matthews KR: **The developmental cell biology of Trypanosoma brucei**. *J Cell Sci* 2005, **118**(Pt 2):283-290.

16. Van Den Abbeele J, Caljon G, De Ridder K, De Baetselier P, Coosemans M: ***Trypanosoma brucei* modifies the tsetse salivary composition, altering the fly feeding behavior that favors parasite transmission**. *PLoS Pathog* 2010, **6**(6):e1000926.
17. Alves-Silva J, Ribeiro JM, Van Den Abbeele J, Attardo G, Hao Z, Haines LR, Soares MB, Berriman M, Aksoy S, Lehane MJ: **An insight into the sialome of *Glossina morsitans morsitans***. *BMC Genomics* 2010, **11**:213.
18. Van Den Abbeele J, Caljon G, Dierick JF, Moens L, De Ridder K, Coosemans M: **The *Glossina morsitans* tsetse fly saliva: general characteristics and identification of novel salivary proteins**. *Insect Biochem Mol Biol* 2007, **37**(10):1075-1085.
19. Mehlitz D, Zillmann U, Scott CM, Godfrey DG: **Epidemiological studies on the animal reservoir of Gambiense sleeping sickness. Part III. Characterization of trypanozoon stocks by isoenzymes and sensitivity to human serum**. *Tropenmed Parasitol* 1982, **33**(2):113-118.
20. Wang T, Cai ZP, Gu XQ, Ma HY, Du YM, Huang K, Voglmeir J, Liu L: **Discovery and characterization of a novel extremely acidic bacterial *N*-glycanase with combined advantages of PNGase F and A**. *Biosci Rep* 2014, **34**(6):e00149.
21. Tarentino AL, Plummer TH, Jr., Maley F: **The release of intact oligosaccharides from specific glycoproteins by endo-beta-N-acetylglucosaminidase H**. *J Biol Chem* 1974, **249**(3):818-824.
22. Royle L, Radcliffe CM, Dwek RA, Rudd PM: **Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions**. *Methods Mol Biol* 2006, **347**:125-143.
23. Navazio L, Miuzzo M, Royle L, Baldan B, Varotto S, Merry AH, Harvey DJ, Dwek RA, Rudd PM, Mariani P: **Monitoring endoplasmic reticulum-to-Golgi traffic of a plant calreticulin by protein glycosylation analysis**. *Biochemistry* 2002, **41**(48):14141-14149.
24. Kozak RP, Tortosa CB, Fernandes DL, Spencer DI: **Comparison of procainamide and 2-aminobenzamide labeling for profiling and identification of glycans by liquid chromatography with fluorescence detection coupled to electrospray ionization-mass spectrometry**. *Anal Biochem* 2015, **486**:38-40.
25. **NetNGlyc 1.0 Server** [http://www.cbs.dtu.dk/services/NetNGlyc/]
26. Patel NY, Youdeowei A, Odhiambo TR: **The composition of the salivary gland secretion of the tsetse, *Glossina morsitans morsitans* Westwood 1850 (Diptera: Glossinidae)**. *Int J Trop Insect Sci* 1981, **1**(4):383-387.
27. Maley F, Trimble RB, Tarentino AL, Plummer TH, Jr.: **Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases**. *Anal Biochem* 1989, **180**(2):195-204.
28. Casas-Sánchez A, Perally S, Ramaswamy R, Haines LR, Rose C, Yunta C, Aguilera-Flores M, Lehane MJ, Almeida IC, Boulanger MJ *et al*: **The crystal structure and localization of *Trypanosoma brucei* invariant surface glycoproteins suggest a more permissive VSG coat in the tsetse-transmitted metacyclic stage**. *bioRxiv 477737* 2018.
29. Mondragon-Shem K, Wongtrakul-Kish K, Kozak RA, Yan S, Wilson I, Paschinger K, Rogers ME, Spencer DI, Acosta Serrano A: **Insights into the salivary *N*-glycome of *Lutzomyia longipalpis*, vector of visceral leishmaniasis**. *bioRxiv 132746* 2020.
30. International Glossina Genome I: **Genome sequence of the tsetse fly (*Glossina morsitans*): vector of African trypanosomiasis**. *Science* 2014, **344**(6182):380-386.
31. Attardo GM, Abd-Alla AMM, Acosta-Serrano A, Allen JE, Bateta R, Benoit JB, Bourtzis K, Caers J, Caljon G, Christensen MB *et al*: **Comparative genomic analysis of six *Glossina* genomes, vectors of African trypanosomes**. *Genome Biol* 2019, **20**(1):187.

32. Rogerson E, Pelletier J, Acosta-Serrano A, Rose C, Taylor S, Guimond S, Lima M, Skidmore M, Yates E: **Variations in the peritrophic matrix composition of heparan sulphate from the tsetse fly, *Glossina morsitans morsitans***. *Pathogens* 2018, **7**(1).
33. Tiemeyer M, Selleck SB, Esko JD: **Arthropoda**. In: *Essentials of Glycobiology.* Edited by nd, Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME. Cold Spring Harbor (NY); 2009.
34. Staudacher E, Altmann F, Marz L, Hard K, Kamerling JP, Vliegenthart JF: **Alpha 1-6(alpha 1-3)-difucosylation of the asparagine-bound *N*-acetylglucosamine in honeybee venom phospholipase A2**. *Glycoconj J* 1992, **9**(2):82-85.
35. Ellis JA, Shapiro SZ, Moi-Yoi O, Moloo SK: **Lesions and saliva-specific antibody responses in rabbits with immediate and delayed hypersensitivity reactions to the bites of *Glossina morsitans centralis***. *Vet Pathol* 1986, **23**:661-667.
36. Haddow JD, Poulis B, Haines LR, Gooding RH, Aksoy S, Pearson TW: **Identification of major soluble salivary gland proteins in teneral *Glossina morsitans morsitans***. *Insect Biochem Mol Biol* 2002, **32**(9):1045-1053.
37. Caljon G, De Ridder K, De Baetselier P, Coosemans M, Van Den Abbeele J: **Identification of a tsetse fly salivary protein with dual inhibitory action on human platelet aggregation**. *PLoS One* 2010, **5**(3):e9671.
38. Mabille D, Caljon G: **Inflammation following trypanosome infection and persistence in the skin**. *Curr Opin Immunol* 2020, **66**:65-73.
39. Ramamoorthi N, Narasimhan S, Pal U, Bao F, Yang XF, Fish D, Anguita J, Norgard MV, Kantor FS, Anderson JF *et al*: **The Lyme disease agent exploits a tick protein to infect the mammalian host**. *Nature* 2005, **436**(7050):573-577.
40. Schuijt TJ, Hovius JW, van Burgel ND, Ramamoorthi N, Fikrig E, van Dam AP: **The tick salivary protein Salp15 inhibits the killing of serum-sensitive *Borrelia burgdorferi* sensu lato isolates**. *Infect Immun* 2008, **76**(7):2888-2894.
41. Kolb P, Vorreiter J, Habicht J, Bentrop D, Wallich R, Nassal M: **Soluble cysteine-rich tick saliva proteins Salp15 and Iric-1 from *E. coli***. *FEBS Open Bio* 2015, **5**:42-55.
42. Cao-Lormeau VM: **Dengue viruses binding proteins from *Aedes aegypti and Aedes polynesiensis* salivary glands**. *Virol J* 2009, **6**:35.
43. Conway MJ, Londono-Renteria B, Troupin A, Watson AM, Klimstra WB, Fikrig E, Colpitts TM: ***Aedes aegypti* D7 saliva protein inhibits dengue virus infection**. *PLoS Negl Trop Dis* 2016, **10**(9):e0004941.
44. Schleicher TR, Yang J, Freudzon M, Rembisz A, Craft S, Hamilton M, Graham M, Mlambo G, Tripathi AK, Li Y *et al*: **A mosquito salivary gland protein partially inhibits *Plasmodium sporozoite* cell traversal and transmission**. *Nat Commun* 2018, **9**(1):2908.
45. Vazquez-Mendoza A, Carrero JC, Rodriguez-Sosa M: **Parasitic infections: a role for C-type lectins receptors**. *Biomed Res Int* 2013, **2013**:456352.
46. Martinez-Pomares L: **The mannose receptor**. *J Leukoc Biol* 2012, **92**(6):1177-1186.
47. Koppel EA, Ludwig IS, Hernandez MS, Lowary TL, Gadikota RR, Tuzikov AB, Vandenbroucke-Grauls CM, van Kooyk Y, Appelmelk BJ, Geijtenbeek TB: **Identification of the mycobacterial carbohydrate structure that binds the C-type lectins DC-SIGN, L-SIGN and SIGNR1**. *Immunobiology* 2004, **209**(1-2):117-127.
48. Argueta-Donohue J, Wilkins-Rodriguez AA, Aguirre-Garcia M, Gutierrez-Kobeh L: **Differential phagocytosis of *Leishmania mexicana* promastigotes and amastigotes by monocyte-derived dendritic cells**. *Microbiol Immunol* 2016, **60**(6):369-381.

683 49. Guo Y, Feinberg H, Conroy E, Mitchell DA, Alvarez R, Blixt O, Taylor ME, Weis WI, Drickamer K: **Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR**. *Nat Struct Mol Biol* 2004, **11**(7):591-598.
686 50. Evans-Osses I, de Messias-Reason I, Ramirez MI: **The emerging role of complement lectin pathway in trypanosomatids: molecular bases in activation, genetic deficiencies, susceptibility to infection, and complement system-based therapeutics**. *ScientificWorldJournal* 2013, **2013**:675898.
690 51. Gout E, Garlatti V, Smith DF, Lacroix M, Dumestre-Perard C, Lunardi T, Martin L, Cesbron JY, Arlaud GJ, Gaboriaud C *et al*: **Carbohydrate recognition properties of human ficolins: glycan array screening reveals the sialic acid binding specificity of M-ficolin**. *J Biol Chem* 2010, **285**(9):6612-6622.
694 52. Andrews L, Laughinghouse A, Sina BJ: **Lectin binding characteristics of male and female salivary gland proteins of *Anopheles gambiae*: Identification and characterization of female specific glycoproteins**. *Insect Biochem Mol Biol* 1997, **27**(2):159-166.
697 53. Poehling HM, Meyer W: **Esterases and glycoproteins in the salivary glands of *Anopheles stephensi***. *Insect Biochem* 1980, **10**:189-198.
699 54. Arca B, Lombardo F, Francischetti IM, Pham VM, Mestres-Simon M, Andersen JF, Ribeiro JM: **An insight into the sialome of the adult female mosquito *Aedes albopictus***. *Insect Biochem Mol Biol* 2007, **37**(2):107-127.
702 55. Volf P, Tesarova P, Nohynkova EN: **Salivary proteins and glycoproteins in phlebotomine sandflies of various species, sex and age**. *Med Vet Entomol* 2000, **14**(3):251-256.

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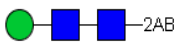
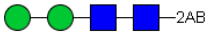
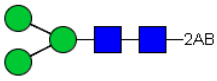
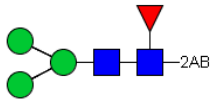
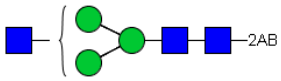
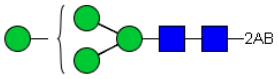
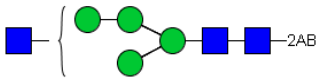
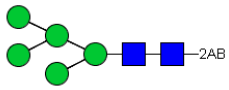
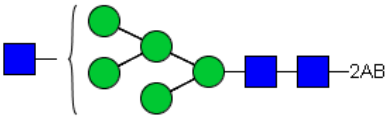
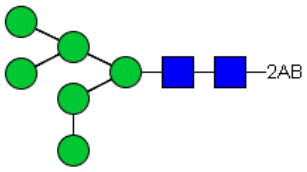
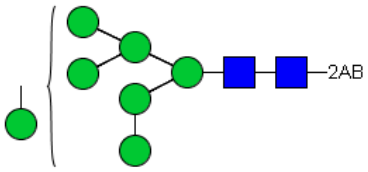
705 Table 1. Exoglycosidase digestion of 2-AB-labelled glycans released from teneral fly saliva by PNGase F.
706 [a]Numbers are percentage areas; [b]Digestion product only. [c]Complete digestion after incubation with bkF +
707 JBM is likely due to the presence of contaminating (~0.2%) β-*N*-acetylglucosaminidase activity in
708 commercial bfk enzyme. Undig, whole glycan pool before digestion; GUH, *Streptococcus pneumonia* in *E*
709 *coli* β-N-acetylglucosaminidase; JBM, Jack bean α-Mannosidase; bkF, Bovine kidney α-Fucosidase. The
710 symbols for glycan structures are adopted from the Consortium for Functional Glycomics: Mannose,
711 green circle; *N*-Acetylglucosamine, blue square; Fucose, red triangle.
712

HPLC Peak Id	GU (2-AB)	Structure	Enzymes used [a]			
			Undig	GUH	JBM	bkF + JBM
A	2.55	[b] 	0.00	0.00	90.64	91.44
1	3.84		1.59	2.85	0.00	0.00
2	4.34		55.35	52.06	0.00	0.00

3	4.83		2.23	4.05	0.00	0.00
4	4.96		4.06	0.00	3.32	0.00
5	5.18		3.09	5.55	0.00	0.00
6	5.74		1.53	0.00	0.46	0.00
7	6.17		8.41	10.04	0.00	0.00
8	6.66		1.76	0.00	0.15	0.00
9	7.09		5.94	6.38	0.00	0.00
10	7.97		7.70	8.12	0.00	0.00
11	8.71		1.37	2.87	0.00	0.00

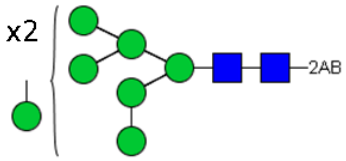
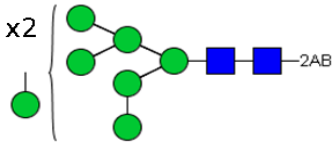
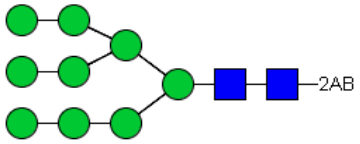
						
12	8.84		2.13	3.08	0.00	0.00
13	9.54		4.86	5.01	0.00	0.00

Table 2. Comparison of relative abundance of released *N*-glycans present in saliva samples from teneral (unfed), naïve and *T. brucei*-infected flies.

HPLC Peak Id	Teneral		Naïve		Infected	
	GU	% Area	GU	% Area	GU	% Area
1	3.84	1.59	3.84	0.51	3.84	0.83
2	4.34	55.35	4.34	54.67	4.34	53.55
3	4.83	2.23	4.83	2.78	4.83	3.80
4	4.96	4.06	4.96	10.57	4.96	10.23
5	5.18	3.09	5.18	1.48	5.18	1.70
6	5.74	1.53	5.74	1.67	5.74	1.74
7	6.17	8.41	6.17	8.17	6.17	8.86
8	6.66	1.76	6.66	1.39	6.66	1.28
9	7.06	5.94	7.06	3.93	7.06	3.55
10	7.97	7.70	7.97	5.15	7.98	4.87
11	8.71	1.37	8.72	2.27	8.72	1.97
12	8.84	2.13	8.84	1.35	8.84	1.33
13	9.54	4.86	9.54	6.06	9.54	6.28