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Insights into the salivary *N*-glycome of *Lutzomyia longipalpis*, vector of visceral leishmaniasis

Karina Mondragon-Shem[1,7], Katherine Wongtrakul-Kish[2,5,7], Radoslaw P. Kozak[2], Shi Yan[3,6], Iain B. H. Wilson[3], Katharina Paschinger[3], Matthew E. Rogers[4], Daniel I. R. Spencer[2] & Alvaro Acosta-Serrano[1]✉

During *Leishmania* transmission sand flies inoculate parasites and saliva into the skin of vertebrates. Saliva has anti-haemostatic and anti-inflammatory activities that evolved to facilitate bloodfeeding, but also modulate the host's immune responses. Sand fly salivary proteins have been extensively studied, but the nature and biological roles of protein-linked glycans remain overlooked. Here, we characterised the profile of *N*-glycans from the salivary glycoproteins of *Lutzomyia longipalpis*, vector of visceral leishmaniasis in the Americas. In silico predictions suggest half of *Lu. longipalpis* salivary proteins may be *N*-glycosylated. SDS-PAGE coupled to LC–MS analysis of sand fly saliva, before and after enzymatic deglycosylation, revealed several candidate glycoproteins. To determine the diversity of *N*-glycan structures in sand fly saliva, enzymatically released sugars were fluorescently tagged and analysed by HPLC, combined with highly sensitive LC–MS/MS, MALDI-TOF–MS, and exoglycosidase treatments. We found that the *N*-glycan composition of *Lu. longipalpis* saliva mostly consists of oligomannose sugars, with $Man_5GlcNAc_2$ being the most abundant, and a few hybrid-type species. Interestingly, some glycans appear modified with a group of 144 Da, whose identity has yet to be confirmed. Our work presents the first detailed structural analysis of sand fly salivary glycans.

Sand flies are small insects that can transmit bacteria and viruses[1,2], but are known mainly as vectors of leishmaniasis, a disease that threatens 350 million people worldwide[3]. When female sand flies feed, they inject a saliva comprised of molecules that facilitate the ingestion of blood, and modulate the host immune system and pathogen transmission[4–6]. These effects have led researchers to explore the potential of insect salivary molecules as markers of biting exposure[5,7] (to determine risk of disease), or even as components of vaccines against leishmaniasis[8]. Of the many types of molecules that make up saliva, most research has focused on the proteins; here, we have investigated the glycans that modify these proteins.

In most eukaryotic cells, the addition of glycans to proteins is a highly conserved and diverse post-translational modification. The most common types of protein-linked glycans are *N*-linked (attached to asparagine residues in the sequon Asn-X-Thr/Ser), and *O*-linked (attached to serine or threonine residues). Glycoconjugates display a wide range of biological roles, from organism development to immune system functions against pathogens[9]. One study has addressed the types and roles of glycans in insects using the model fruit fly, *Drosophila melanogaster*. In this species, biological functions have been attributed to different glycan classes, such as morphology and locomotion (*N*-linked glycans), or cell interaction and signalling (*O*-linked glycans)[10].

[1]Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK. [2]Ludger Ltd., Culham Science Centre, Oxfordshire OX14 3EB, UK. [3]Department of Chemistry, University of Natural Resources and Life Sciences, 1190 Vienna, Austria. [4]Department of Disease Control, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK. [5]Present address: Australian Research Council Centre of Excellence for Nanoscale Biophotonics, Macquarie University, Sydney, Australia. [6]Present address: Institute of Parasitology, Department of Pathobiology, University of Veterinary Medicine, A-1210 Vienna, Austria. [7]These authors contributed equally: Karina Mondragon-Shem and Katherine Wongtrakul-Kish. ✉email: alvaro.acosta-serrano@lstmed.ac.uk

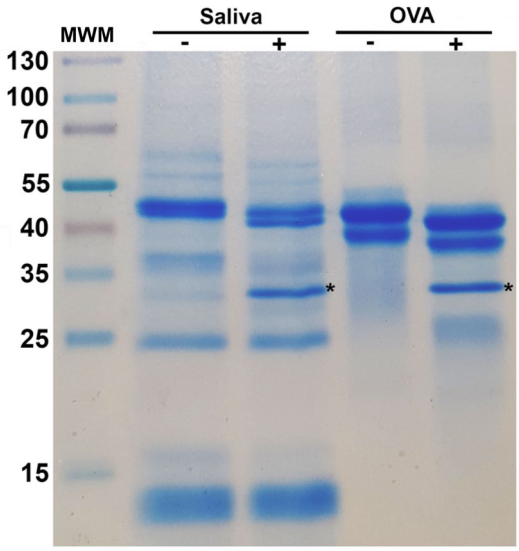


Figure 1. Enzymatic cleavage of *Lu. longipalpis* salivary glycoproteins with PNGase F. 10 µg of salivary proteins were incubated overnight with (+) and without (-) PNGase F to cleave *N*-glycans. Samples were resolved on a 12% SDS-PAGE gel and Coomassie-stained. Egg albumin (OVA) was used as a positive control. *MWM* molecular weight marker. *PNGase F enzyme.

Glycans may have special relevance in the saliva of medically important arthropods, because of the fundamental role this biological fluid plays during pathogen transmission. For instance, African trypanosomes, tick-borne pathogens, arboviruses and malaria are all harboured in the salivary glands of their respective vectors, and are co-transmitted with saliva through the bite. In contrast, *Leishmania* parasites are transmitted by regurgitation from the fly's midgut, where infectious stages reside, and contact with saliva occurs in the host at the bite site[11]. People living in leishmaniasis-endemic regions are constantly exposed to the saliva of uninfected sand flies, triggering immune responses that may later influence parasite infection[12]. The immunogenicity of salivary glycan structures and their interaction with specific immune cells could have different effects for each disease.

There are some reports describing the presence of salivary glycoproteins in sand flies through in silico and blotting analyses[13–19]; however, to our knowledge no detailed structural studies have been published to date. Therefore, we set out to identify the salivary glycoproteins in the sand fly vector species *Lutzomyia longipalpis*, and structurally characterise their *N*-glycan conjugates. We further discuss their implications for insect blood-feeding as well as vector-host interactions.

Results

Identification of *Lutzomyia longipalpis* salivary glycoproteins. To determine the degree of *N*-glycosylation, an in silico analysis was carried out on 42 salivary proteins previously reported in *Lu. longipalpis*[4,20] to predict protein *N*-glycosylation sites using the NetNGlyc server (https://www.cbs.dtu.dk/services/NetNGlyc/). This revealed 48% of the commonly known salivary proteins contain conventional *N*-glycosylation sites (Supplementary Table S1). However, it is important to note this list only includes proteins available on the NCBI database as studies published to date have focused on major secreted proteins, and no deep sequencing has been carried out for salivary glands of this sand fly species.

To accompany the in silico dataset, we carried out our own analysis of the sand fly salivary proteins (Supplementary Fig. S1). First, *Lu. longipalpis* salivary glands were dissected and individually pierced to release saliva. Subsequent Coomassie blue SDS-PAGE analysis showed several protein bands ranging from ~ 10 to 100 kDa (Fig. 1). To identify which proteins were glycosylated, samples were analysed before and after treatment with Peptide-N-Glycosidase F (PNGase F), which cleaves high-mannose, hybrid and complex *N*-linked glycans. Treatment with PNGase F resulted in molecular mass shifts and migration of several protein bands, consistent with the widespread removal of *N*-glycans from the salivary glycoproteins (Fig. 1). De-glycosylation was also confirmed by transferring proteins to PVDF membrane and blotting with Concanavalin A (ConA) lectin, which binds specifically to terminal mannose residues on glycoproteins[21] (Supplementary Fig. S2).

For LC–MS/MS based glycoprotein identification, the major deglycosylated protein bands (Supplementary Fig. S3) were excised from the gel and sent to the University of Dundee Fingerprints Proteomics Facility. From the resulting list of 191 identified proteins, we excluded those without recognizable glycosylation sequons (as determined by NetNGlyc), obtaining a final list of 43 potentially *N*-glycosylated protein candidates (Supplementary Table S2). Fourteen of these potential glycoproteins were also identified in our initial in silico analysis (Supplementary Table S1), including LJM11, LJM111 and LJL143, which have been proposed as potential vaccine components against *Leishmania* infection[4]. Using the InterProScan tool to identify conserved protein domains, family distributions (Supplementary Fig. S4) show five of the candidates belonging to the actin family, while others like tubulin, 5′ nucleotidase, peptidase M17 and the major royal jelly protein (yellow protein) are represented

by two proteins each. After Blast2GO analysis, the "molecular function breakdown" suggested that 44% of the candidate glycoproteins are involved in binding, including 'small molecule binding' and 'carbohydrate derivative binding' (Supplementary Fig. S4). We also used the DeepLoc server to predict protein subcellular localisation and solubility of the proteins identified in Table S2. The results suggest 85% of candidate glycoproteins are soluble, and 10 proteins are both extracellular and soluble (Supplementary Table S2).

Salivary glycoproteins from *Lu. longipalpis* are mainly modified with mannosylated *N*-glycans. Next, we determined the *N*-glycome modifying the salivary proteins of *Lu. longipalpis*. The presence of mannosylated *N*-glycan structures on salivary glycoproteins was suggested by the results of a lectin blot using Concanavalin A, and to confirm these results, we next determined the *N*-glycome of salivary glycoproteins of *Lu. longipalpis*.

The oligosaccharides were released by PNGase F followed by derivatization with procainamide[22] which allowed fluorescence detection following hydrophilic interaction liquid chromatography (HILIC) and provided increased signal intensity in MS and MS/MS analysis[22]. Overall, we identified 14 different structures (Table 1), elucidated from ten separate compositions due to the presence of isomeric glycans.

Most oligosaccharides are of the high mannose type (82% of the *N*-glycome), with the $Man_5GlcNAc_2$-Proc glycan with *m/z* $[727.81]^{2+}$, being the most abundant species (21.16 min; GU 6.00, Fig. 2). In addition, few hybrid-type species (with a retention time of 15.12–17.24 min) were detected, containing either an α1-6 core fucose residue linked to the reducing GlcNAc or not fucosylated, or a single terminal LacNAc motif (Fig. 2).

All major glycan structures were characterised using positive ion MS (Fig. 3A) and MS/MS fragmentation spectra. An example of structural elucidation using MS/MS fragmentation spectrum is shown for the major glycan species $Man_5GlcNAc_2$-Proc, with *m/z* $[727.82]^{2+}$ (Fig. 3B) while the remaining are mainly represented by hybrid-type glycans, either a trimannosyl modified with a Fuc residue on the chitobiose core, or paucimannosidic structures containing an unknown modification of 144 Da (see below).

Although PNGase F is highly effective in cleaving *N*-linked glycans, its activity is blocked by the presence of core fucose residues with an α1-3 linkage found in non-mammalian glycans. Therefore, we also treated our samples with PNGase A, which cleaves all glycans between the innermost GlcNAc and the asparagine independent of core linkages[23]. No differences were observed in chromatograms yielded from both enzymes (Supplementary Fig. S5), indicating all core fucosylation is likely to be α1-6-linked.

MALDI-TOF–MS analysis reveals a series of sand fly salivary glycans with unidentified modifications of 144 Da. A more detailed analysis of the saliva by MALDI-TOF MS of pyridylaminated glycans revealed not only the major oligomannosidic species, but also suggested the existence of a series of glycans containing an unidentified structure. This modification was mainly found in two isomeric glycans: one with an RP-HPLC retention time of 25.0 min and the other of 26.5 min (Supplementary Fig. S6). The two isomers have a *m/z* 1,295.50, which corresponds to a pyridylaminated $Man_4GlcNAc_2$ glycan carrying a modification of 144 Da. This was confirmed by treatment with Jack bean α-mannosidase, which resulted in a loss of 2 and 3 hexoses (Fig. 4) for each isomer, respectively. Interestingly, this modification seems to be located in different positions in the two structures, and in both cases this modification was lost after treatment with 48% aqueous hydrofluoric acid (aq.HF) (Fig. 4, and Table 2).

Susceptibility to aq.HF is a hallmark of phosphoester, galactofuranose and some fucose modifications, but none of these are obviously compatible with a 144 Da modification. Based on this data, a re-assessment of the data with the procainamide-labelled glycans also revealed a total of four structures carrying this modification (Peak 4, 5, 7 and 8, Table 1); however, due to the very low abundance of these glycans we were unable to determine their chemical nature. Additionally, the potential for anionic modifications of *N*-glycans was explored by both glycomic workflows, but limitations in spectral quality and sample amount prevented a definitive characterisation.

No *O*-linked glycans found in sand fly saliva. In silico predictions using the NetOGlyc 4.0[24] server suggest that 85% our 191 identified salivary proteins have putative *O*-glycosylation sites (Supplementary Table S3). Sand fly saliva was subjected to reductive β-elimination to release *O*-glycans from the de-*N*-glycosylated proteins. Separation using porous graphitized carbon chromatography coupled with negative ion mode ESI–MS did not detect any *O*-glycans in the sample (Supplementary Fig. S8), either due to their absence, low abundance or low mass.

Discussion

Sand fly saliva has important implications both for the insect and the vertebrate host[4]. *Lu. longipalpis* salivary proteins and their biological roles have been well studied[4,20]; however, the sugars that modify these proteins have not been characterised in detail. Most work on sand fly salivary glycans comes from in silico analyses[13–15,17,18,25] and lectin blotting. They were first reported by Volf et al[19], who used lectins to detect mannosylated *N*-type glycans. Mejia et al[16] reported high mannose glycans in *Lu. longipalpis* saliva, with some potential hybrid-type structures (also based on lectin specificity). However, results from lectin-based methods should be interpreted with care as detection controls have not always been included in these studies, and results can be highly dependent on glycan abundance in samples and specific protocols. Our work is the first time that a mass spectrometry approach has been used to study the salivary *N*-linked glycans of *Lu. longipalpis*, providing detailed information about their structures and relative abundances. We found that sand fly salivary glycoproteins consist mainly of oligomannose glycans (ranging from the core $Man_3GlcNAc_2$ to $Man_9GlcNAc_2$), with some hybrid-type (e.g. fucosylated) structures. Additionally, this is the first report of a 144 Da (unknown) modification present in some salivary glycans. Our results provide new insights into how these structures could be recognised by vertebrate host cells.

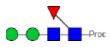
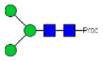
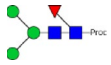
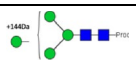
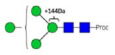
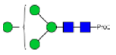
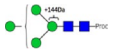
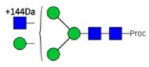
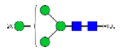
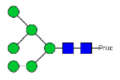
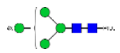
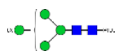
Peak No.	GU	Detected $[M+Proc+2H]^{2+}$	Detected $[M+Proc+H]^{1+}$	Theoretical $[M+Proc+H]^{1+}$	Composition	% Relative Abundance	Proposed Structure
1	3.69	–	1114.48	1114.51	$(Hex)_2(HexNAc)_2(Fucose)_1$ + contaminant	10.31	
2	4.21	–	1130.52	1130.51	$(Hex)_3(HexNAc)_2$ + contaminant	11.35	
3	4.60	638.79	1276.58	1276.57	$(Hex)_3(HexNAc)_2(Fucose)_1$	2.30	
4	4.87	718.8	1436.58	1436.48	$(Hex)_4(HexNAc)_2$	0.41	
5	4.95	718.8	–	1495.64	$(Hex)_4(HexNAc)_2$	0.53	
		748.79	1436.58	1436.48	$(Hex)_1(HexNAc)_1$ + $(Man)_3(GlcNAc)_2$		
6	5.02	646.79	1292.58	1292.56	$(Hex)_4(HexNAc)_2$ + contaminant	8.21	
7	5.15	718.8	–	1436.48	$(Hex)_4(HexNAc)_2$	3.87	
8	5.88	819.31	–	1639.56	$(HexNAc)_1$ + 144 + Hexose + $(Man)_3(GlcNAc)_2$ + Proc	4.16	
9	6.00	727.8	1454.59	1454.61	$(Hex)_2$ + $(Man)_3(GlcNAc)_2$	41.43	
		646.79	1292.58	1292.56	$(Hex)_4(HexNAc)_2$		
10	6.83	808.82	1616.64	1616.67	$(Hex)_3$ + $(Man)_3(GlcNAc)_2$	9.84	
11	7.44	727.23	–	1454.61	$(Hex)_2$ + $(Man)_3(GlcNAc)_2$	0.38	
12	7.72	889.83	–	1778.72	$(Hex)_4$ + $(Man)_3(GlcNAc)_2$	2.77	
13	8.58	970.85	–	1940.77	$(Hex)_5$ + $(Man)_3(GlcNAc)_2$	2.83	
14	9.31	1051.9	–	2102.82	$(Hex)_6$ + $(Man)_3(GlcNAc)_2$	0.90	

Table 1. List of glycan structures present in *Lu. longipalpis* saliva. *GU* glucose units, *Proc* procainamide Green circles, mannose; blue squares, *N*-acetylglucosamine; red triangle, fucose; yellow circles, galactose. Relative abundance values are based on HILIC data (Fig. 2).

In insects, protein glycosylation studies have been carried out primarily on the *Drosophila melanogaster* fly, demonstrating the presence of various carbohydrate structures[10,26,27]. It is generally accepted that *N*-linked type glycoproteins in arthropods are mainly of the high-mannose or paucimannose type, and account for over 90% of glycan complexity in *Drosophila*[10,28]. One of the first indications of the capacity of insects to produce complex type *N*-glycans came from bee venom phospholipase A2, which contains the core α1,3-fucose (an IgE epitope allergenic to humans). Anionic and zwitterionic *N*-glycans with up to three antennae have more recently been found in a range of insects[29–32]. Furthermore, Vandenborre et al.[33] explored glycosylation differences comparing

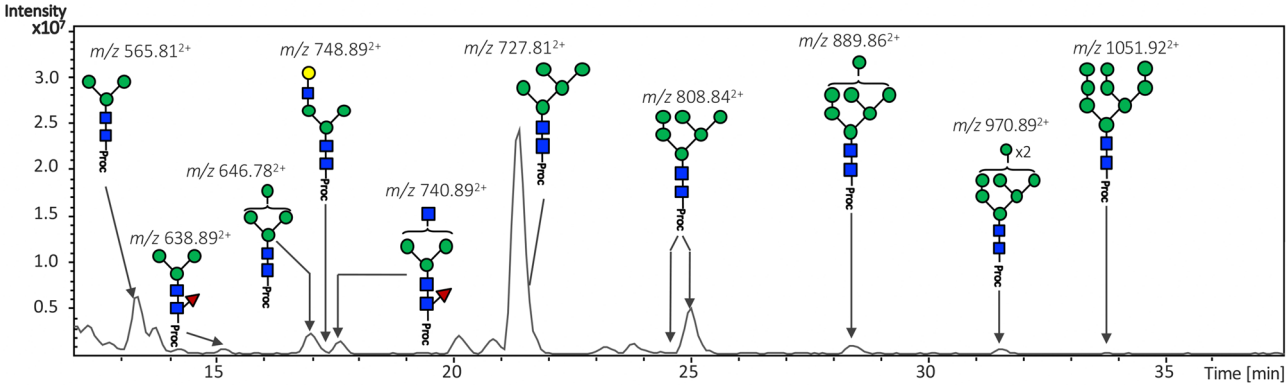


Figure 2. HILIC-LC separation of procainamide labelled *N*-glycans from *Lu. longipalpis*. Sand fly saliva contains mainly oligomannose-type *N*-linked glycans, with $Man_5GlcNAc_2$ being the most abundant structure. Green circle, mannose; yellow circle, galactose; blue square, *N*-acetylglucosamine; red triangle, fucose; *Proc* procainamide.

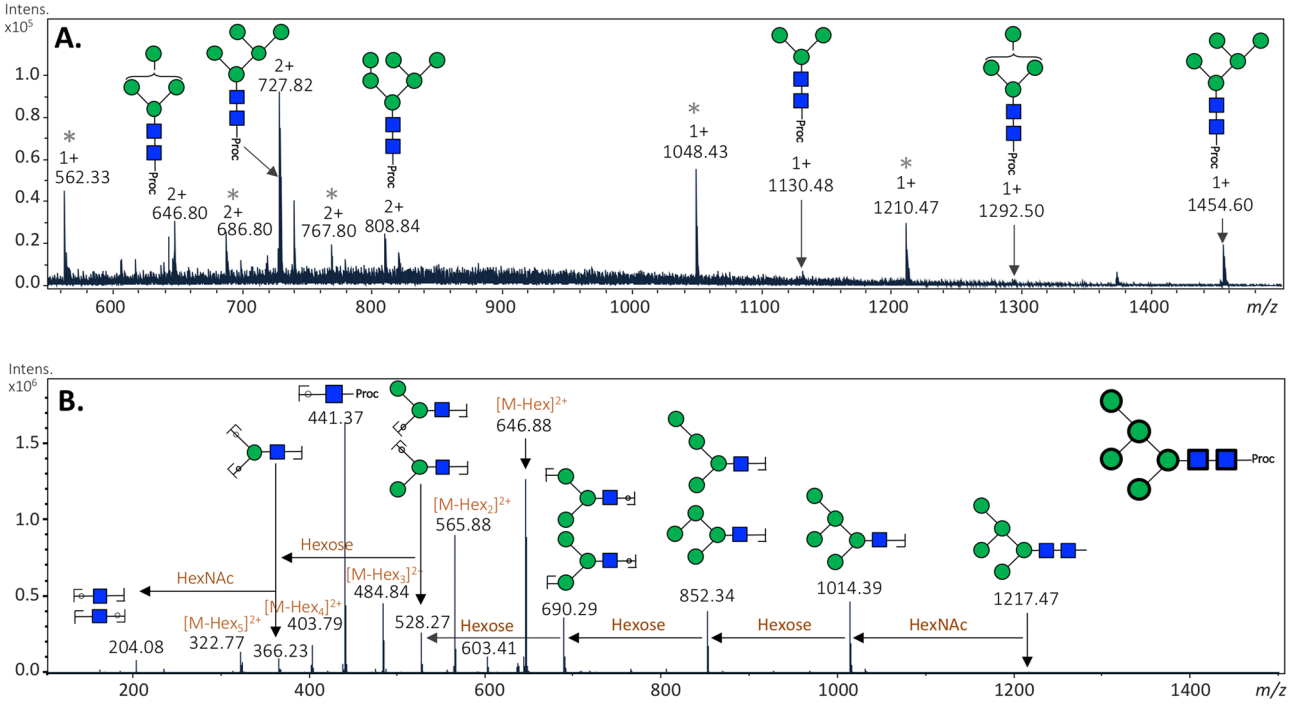


Figure 3. Mass spectrometry analysis of released *N*-glycans from *Lu. longipalpis* salivary glycoproteins. **(A)** Positive-ion mass spectrum profile (*m/z* 540–1,500) of total *N*-glycans. Ion signals are labelled accordingly. The most abundant glycan species ($Hex_5HexNAc_2$–Proc) was also detected as an $[M+H]^{2+}$ ion with a *m/z* of 727.82. See Table 1 for complete glycan assignment. Peaks labelled with an asterisk correspond to glucose homopolymer contaminants from HILIC. **(B)** Positive-ion MS/MS fragmentation spectrum for most abundant *m/z* $[727.8]^{2+}$ corresponding to the composition $Hex_5HexNAc_2$–Proc, proposed as a $Man_5GlcNAc_2$. Green circle, mannose; Blue square, *N*-acetylglucosamine; *Proc* procainamide.

several economically important insects, and found glycoproteins to be involved in a broad range of biological processes such as cellular adhesion, homeostasis, communication and stress response.

Some researchers have predicted the presence of mucins in the mouthparts of bloodfeeders[34,35], proposing their possible role as lubricants to facilitate bloodmeals. Even though *O*-linked glycans have been widely documented in invertebrates, we were unable to detect these sugars in sand fly saliva after reductive β-elimination. This was surprising given that our bioinformatic analysis (NetOGlyc server) predicted the presence of putative *O*-glycosylation sites. The presence of *O*-linked glycans in *Lu. longipalpis* saliva has been suggested through peanut agglutinin and *Vicia villosa* lectin detection[16]; however, it is worth noting that the experiment does not include positive controls or binding inhibition by competitive sugars, so non-specific binding cannot be ruled out. Interestingly, *Lu. longipalpis* midgut mucin-like glycoprotein has been described[36] (with a suggested role in *Leishmania* attachment), showing the capacity of this species to produce *O*-linked glycans (at least in other

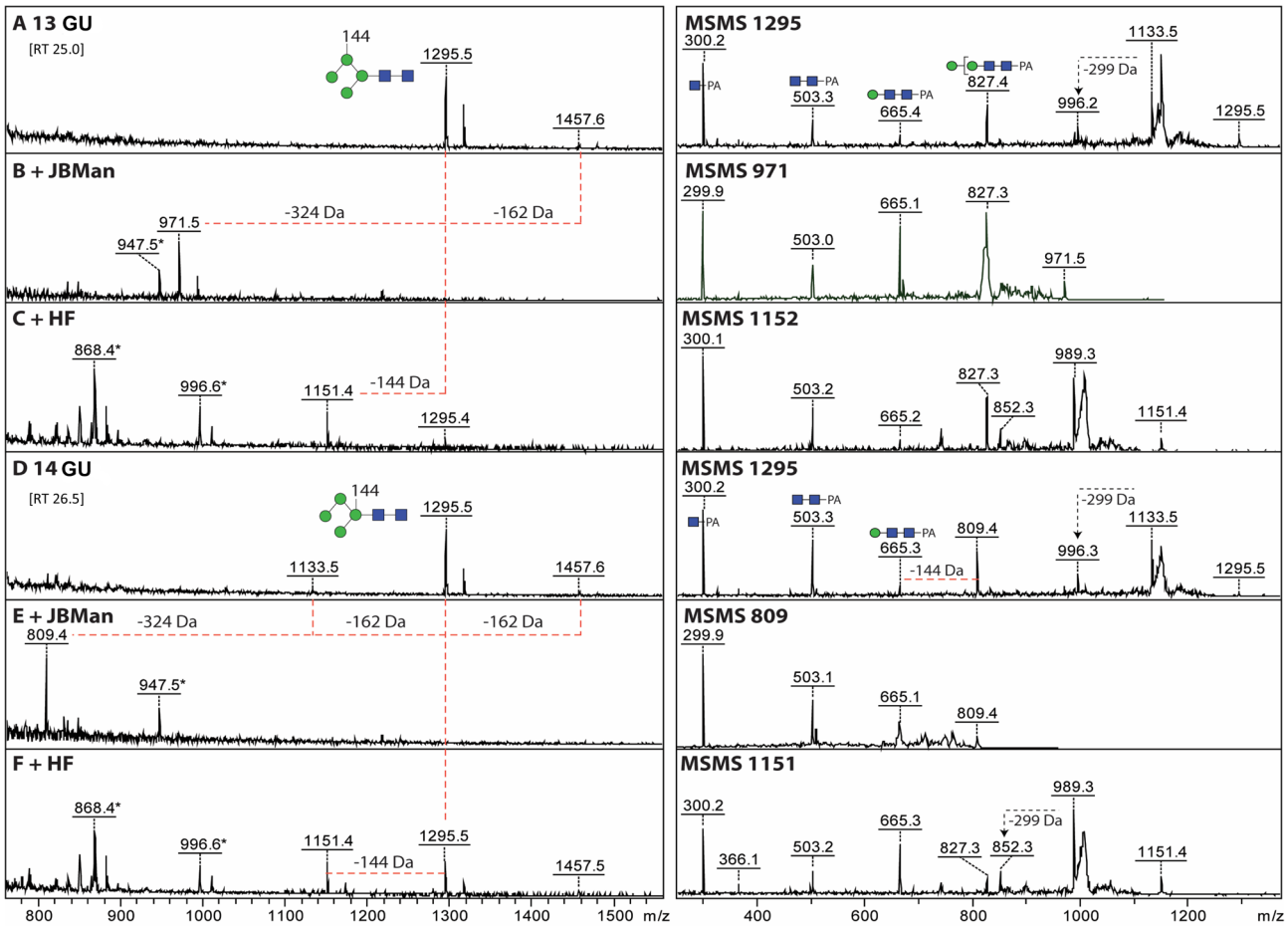


Figure 4. Analysis of sand fly *N*-glycans with an unknown residue. Two late-eluting RP-amide fractions (13 and 14 GU) containing glycans of *m/z* 1,133, 1,295 and 1,457 **(A, D)** were analysed by MALDI-TOF MS and MS/MS before and after jack bean α-mannosidase **(B, E)** or hydrofluoric acid **(C, F)** treatments. The *m/z* 1,295 glycan structures lost either two or three mannose residues after mannosidase treatment, ruling out that terminal α-mannose residues are substituted, but indicating a difference in the isomeric structure. In contrast, upon hydrofluoric acid treatment, incomplete loss of 144 Da was observed. Changes in mass upon mannosidase or HF treatment are indicated and non-glycan impurities annotated with an asterisk. The MS/MS for the original glycans and their digestion products are shown on the right; the differences in relative intensity of the *m/z* 665 and 827 fragments could explain the isomeric *m/z* 1,295 structures with the 144 Da moiety attached to different mannose residues (as shown in panels A and D); key fragments are annotated according to the Symbolic Nomenclature for Glycans, while loss of reducing terminal GlcNAc-PA is indicated by − 299 Da. *PA* 2-aminopyridine, *GU* glucose units, green circle, mannose; blue square, *N*-acetylglucosamine.

Treatment	Isomers	
(RT, min)	25.0	26.5
GU	13	14
No treatment	*m/z* 1,295.5	*m/z* 1,295.5
JBMan	(− 2 Hex)	(− 3 Hex)
48% aq.HF alone	*m/z* 1,151.4 (− 144 Da)	*m/z* 1,151.4 (− 144 Da)
48% aq.HF + 1,3-specific JBMan	*m/z* 989 (-162 (Hex))	No loss observed

Table 2. Summary of treatments of the isomeric structures detected by MALDI-TOF MS (Fig. 4). *JBMan* Jack Bean α-mannosidase, *GU* glucose units, *RT* retention time, *aq.HF* aqueous hydrofluoric acid.

tissues). A variety of *O*-linked glycans are reported for *Drosophila*[37], with important functions such as body development[10,38]. Furthermore, research shows that several *Drosophila*[37] and moth[39] cell lines form mucin-type *O*-glycans. It is worth noting there is no consensus sequence for *O*-glycosylation as in *N*-linked glycosylation, and in silico predictions are unreliable. Interestingly, similar results have been found in *Glossina* (unpublished),

suggesting that these dipterans may not be able to *O*-glycosylate proteins in salivary tissues, or they are below the level of mass spectrometry detection.

A surprising finding in this work were the 144 Da structures modifying some of the salivary glycans (i.e. $Man_4GlcNAc_3$, and two $Man_4GlcNAc_2$ isomers). They were present in very low abundance (< 1%), were located on different mannose residues (as shown by jack bean α-mannosidase digestion), and appeared susceptible to aqueous HF. However, we have yet to confirm the identity and biological role of this modification. A literature search revealed that structures of a 144 Da mass have been found on glycans from other organisms, including bacteria, viruses and sea algae[40–42], but were not further addressed by the authors. One possibility is that these correspond to an anhydrosugar, like 3,6-anhydrogalactose (of 144 Da mass)[43]. Interestingly, work on mosquitoes has shown that these insects are able to produce anionic glycans with sulphate and/or glucuronic modifications that can be tissue specific[29,44]. The glycans identified here carrying this rare 144 Da residue may be another example of such modifications and could play a role specific to their location in sand fly saliva.

Even though every effort was made during salivary gland dissections to obtain saliva with minimal tissue contamination, this cannot be completely avoided. Analysis with the DeepLoc server suggested that although most protein candidates are 'soluble', only some are predicted to be 'extracellular'. Furthermore, some proteins without signal peptide can still be secreted through a non-classical or "unconventional" secretory pathway[47,48]. An alternative way of saliva extraction would be to induce salivation by chemical means like pilocarpine[49–51]; however, this carries its own logistical difficulties considering the amount of saliva needed to detect glycans in such low abundances (even with the highly sensitive techniques we have used here). Another limitation of this work is the low protein profile resolution provided by 1D gel electrophoresis, where we may have missed weaker bands during our selection of proteins for sequencing. Higher protein concentrations and analysis through 2D gel electrophoresis could help us address this issue; nevertheless, we believe our work includes the major proteins in *Lu. longipalpis* saliva, providing a good overview of glycan abundance and composition in this bloodfeeding insect.

The biological role of protein glycosylation in the saliva of sand flies (and other bloodfeeding arthropods) is uncertain. One possibility is that glycans affect salivary protein half-life in the blood once they enter vertebrate host. Another possibility is that these glycans influence other in vivo processes like the interactions between saliva and cell surface carbohydrate recognition domains. For instance, the mannose receptor and DC-SIGN are c-type lectins that recognize mannosylated structures (uncommon in vertebrate cells); they are present on macrophages and dendritic cells, playing a role in both innate and adaptive immune systems[52], making glycans highly relevant in parasitic infection processes. Additionally, the mannose-binding lectin activates the 'lectin pathway' of complement, and has an important role in protection against various pathogens[53]. An example of this was reported in tick saliva, which contains a mannose-binding lectin inhibitor whose activity was shown to be glycosylation-dependent[54].

This, in turn, could be of importance within the context of *Leishmania* infection as both macrophages and dendritic cells have been shown to have critical roles in the initial stages of infection and subsequent dissemination of the parasite inside the vertebrate host[55]. In order for *Leishmania* to survive and multiply inside the host, it must be internalized by macrophages; however, promastigotes appear to avoid the MR receptor during invasion, as it promotes inflammation and can be detrimental to their survival[55]. The saliva of *Lu. longipalpis* can prevent macrophages from presenting *Leishmania* antigens to T cells[56], but these effects are species-specific; in the case of other sand flies like *Phlebotomus papatasi*, saliva inhibits the activation of these cells[57]. Work on a patient-isolated *L. major* strain that causes nonhealing lesions in C57BL/6 mice found that its uptake by dermal-macrophages is MR-mediated[58]. Even though the MR does not play a role in the healing strain, it is an indication that sand fly saliva may be involved in other parasite-macrophage interactions. *Leishmania* also interacts with DC-SIGN (particularly amastigotes and metacyclic promastigotes) and this varies depending on species[59]. It remains to be seen whether mannosylated glycoproteins in saliva impair or facilitate these interactions and their outcomes.

Many sand fly salivary proteins are currently being explored as potential vaccine candidates against *Leishmania*, and knowing the nature of their post-translational modifications is relevant to their activity and efficacy. Several salivary proteins from *Lu. longipalpis* that are being researched as vaccine candidates (e.g. LJM11, LJM17 and LJL143[4]) have potential glycosylation sites (as indicated in the results of our in silico analysis). As recombinant versions of these proteins are normally expressed in non-insect cells[60], care should be taken to ensure the glycoprotein's profile and activity remains the same.

Finally, it is also worth considering the role salivary glycoproteins could play inside the sand flies themselves. Both male and female sand flies rely on plant sugars to survive, and Cavalcante et al. showed that *Lu. longipalpis* ingest saliva while sugar feeding[61]. Lectins (which bind to glycans) represent a major part of a plant's defence system[62], and can cause damage to an insect's midgut when ingested[63]. Salivary glycoconjugates may be potentially recognized by these plant lectins, helping to decrease the damage they can cause. Moreover, the ingestion of saliva during the bloodmeal may impact parasite differentiation in the fly's gut[64]. Furthermore, sand fly-borne viruses use the host cell machinery for replication, which includes the insect glycosylation pathways, before it is transmitted to the vertebrate host. In this context, understanding the glycosylation of insect salivary glands is also relevant to understand their pathogenicity.

Methods

Glycoprotein predictions. The servers NetNGlyc 1.0[65] (https://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0[24,66] (https://www.cbs.dtu.dk/services/NetOGlyc/) were used to predict potential glycosylation sites by examination of the consensus sequences. The DeepLoc 1.0[67] server (https://www.cbs.dtu.dk/services/DeepLoc/index.php) was used to predict location of proteins.

Sand fly salivary gland dissection and extraction of saliva. *Lutzomyia longipalpis* sand flies were obtained from a colony at the London School of Hygiene and Tropical Medicine (UK), which originated in Jacobina (Bahia state), Brazil. Salivary glands were dissected from 5-day old, sugar-fed, uninfected females in sterile PBS (Sigma, St. Louis, US). To harvest saliva, pools of 10 salivary glands were placed on ice, pierced with a needle and then centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatant (pure saliva) was stored at -80 °C. Between 0.5–1 µg of protein per sand fly was obtained from dissections.

SDS polyacrylamide gel electrophoresis and staining. Sand fly saliva (10 µg) was run on a 12.5% polyacrylamide gel, before and after deglycosylation with endoglycosidase PNGase F (New England Biolabs, Massachusetts, US). Gel was stained using InstantBlue Protein stain (Expedeon, California, US). Spectra Multicolor Broad Range Protein Ladder (ThermoFisher, UK) was used as molecular weight marker.

Concanavalin A blots. Saliva samples, before and after treatment with PNGase F (New England Biolabs, US) were run on a 12.5% polyacrylamide gel under standard conditions, transferred onto a PVDF membrane (Fisher Scientific, UK), and blocked with 1% BSA (Sigma, St. Louis, US) in PBS-Tw 20 (Sigma, St. Louis, US) overnight at 4 °C. Membrane was incubated with 1 µg/ml biotinylated Concanavalin A (ConA) lectin (Vector Labs, Peterborough, UK) for 1 h at room temperature. After washing, the membrane was incubated with 1:100,000 streptavidin-HRP (Vector Labs, Peterborough, UK). SuperSignal West Pico Chemiluminescent substrate (ThermoFisher, Massachusetts, US) was used to detect the bands. Egg albumin (Sigma, St. Louis, US), a highly mannosylated *N*-linked glycoprotein[68], was used as positive control.

Mass spectrometry analysis. To identify the glycoproteins that were susceptible to PNGase F, bands of interest were sliced from the gel and sent to the Dundee University Fingerprints Proteomics Facility. Briefly, the excised bands were subjected to in-gel trypsination then alkylated with iodoacetamide. The resultant peptides were then analysed via liquid chromatography- tandem mass spectrometry (LC–MS/MS) in a Thermo LTQ XL Linear Trap instrument equipped with a nano-LC. Tandem MS data were searched against the *Lu. longipalpis* database downloaded from VectorBase (https://www.vectorbase.org/proteomes) using the Mascot (version 2.3.02, Matrix Science, Liverpool) search engine. Search parameters were performed as described in elsewhere[69]. For in-solution data, the false discovery rate was filtered at 1%, and individual ion scores ≥ 30 were considered to indicate identity or extensive homology ($p < 0.05$).

Enzymatic release of *N*-linked glycans. The *N*-glycans from sand fly saliva were released by in-gel deglycosylation using PNGase F as described by Royle et al.[70]. For deglycosylation using PNGase A, 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. Samples were dried by vacuum centrifugation and the tryptic peptide mixture was incubated with PNGase A in 100 mM citrate/phosphate buffer (pH 5.0) for 16 h at 37°C[71]. Samples were separated from protein and salts using LudgerClean Protein Binding Plate (Ludger Ltd., Oxfordshire, UK). All wells were flushed with extra water to ensure full recovery and then dried by vacuum centrifugation prior to fluorescent labelling.

Fluorescent labelling and purification of released *N*-glycans. Released *N*-glycans were fluorescently labelled via reductive amination reaction with procainamide using a Ludger Procainamide Glycan Labelling Kit containing 2-picoline borane (Ludger Ltd.). The released glycans were incubated with labelling reagents for 1 h at 65 °C. The procainamide labelled glycans were cleaned up using LudgerClean S Cartridges (Ludger Ltd) and eluted with water (1 mL). Samples were evaporated under high vacuum and re-suspended in water prior to use.

ESI-LC–MS and ESI-LC–MS/MS analysis of procainamide-labelled *N*-glycans. Procainamide labelled samples were analysed by ESI-LC–MS in positive ion mode. 25 µL of each sample were injected onto an ACQUITY UPLC BEH-Glycan 1.7 µm, 2.1 × 150 mm column at 40 °C on the Dionex Ultimate 3000 UHPLC attached to a Bruker Amazon Speed ETD (Bruker, UK). The running conditions used were: solvent A was 50 mM ammonium formate pH 4.4; solvent B was acetonitrile (acetonitrile 190 far UV/gradient quality; Romil #H049). 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). The Amazon Speed settings were the same as described in[72] except that precursor ions were released after 0.2 min and scanned in enhanced resolution within a mass range of 200–1,500 m/*z* (target mass, 900 m/*z*).

Release of *O*-linked glycans. Saliva samples underwent reductive β-elimination to release *O*-glycans after PNGase F treatment. Briefly, samples were diluted in 0.05 M sodium hydroxide and 1.0 M sodium borohydride at a temperature of 45 °C with an incubation time of 14–16 h followed by solid-phase extraction of released *O*-glycans[73]. *O*-glycans were analysed using PGC-LC coupled to negative ion ESI–MS/MS[74] alongside bovine fetuin *O*-glycans as a positive control.

MALDI-TOF analysis of aminopyridine-labelled glycans. Sand fly salivary glycans were released according to previous procedures and labelled with PA (aminopyridine) as described elsewhere[75], prior to RP-HPLC and analysis by MALDI-TOF MS using a Bruker Daltonics Autoflex Speed instrument (Hykollari). Aliquots of samples were treated with Jack bean α-mannosidase (Sigma), α-1,3 mannosidase and 48% aqueous

hydrofluoric acid (aq.HF); the latter under control conditions releases phospho(di)esters, phosphonate, α1,3-fucose and galactofuranose groups. Dried glycan fractions were redissolved in 3 µL of aq.HF on ice (in the cold room) for 36 h prior to repeated evaporation. The digests were re-analysed using MALDI-TOF MS and MS/MS. Spectra were annotated by comparison to previous data on insect N-glycomes in terms of monosaccharide composition (Fx Hy Nz), using retention time, manual interpretation, exoglycosidase treatment results and LIFT fragmentation analysis.

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Author contributions

Designed experiments (K.M.S., K.W.K., D.S., A.A.-S.), performed experiments (K.M.S., K.W.K., S.Y., R.K.) and analyzed the data (K.M.S., K.W.K., S.Y., I.W., K.P., R.K., M.E.R., A.A.-S.), wrote the manuscript (K.M.S., K.W.K., A.A.-S.). All authors reviewed and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to A.A.-S.

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