Proteomics Informed by Transcriptomics for a Qualitative and Quantitative Analysis of the Sialoproteome of Ornithodoros Moubata Adult Ticks

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Research Article

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Abstract

Background

The argasid tick *Ornithodoros moubata* is the main vector in mainland Africa of the African swine fever virus and the spirochete *Borrelia duttoni*, which causes human relapsing fever. Elimination of *O. moubata* populations would contribute to the prevention and control of these two severe diseases. The development of anti-tick vaccines is an eco-friendly and sustainable method for the elimination of tick populations. The tick saliva forms part of the tick-host interface and knowing its composition is key for the identification and selection of vaccine candidate antigens. The aim of the present work is to expand the data on the saliva proteome composition of *O. moubata* adult ticks, particularly of female ticks, since a more in-depth knowledge of the *O. moubata* sialome will allow identifying and selecting novel salivary antigens as targets for tick vaccines.

Methods

We have analysed samples of female and male saliva using two different mass spectrometry approaches: data-dependent acquisition LC-MS/MS and sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS). To maximise the number of protein identifications, a proteomics informed by transcriptomics (PIT) analysis was applied using the *O. moubata* salivary transcriptomic dataset previously obtained by RNAseq.

Results

The SWATH-MS proved to be superior to LC-MS/MS in the study of female saliva since it increased by 60% the number of identified proteins, enhanced the reproducibility of the results and provided a quantitative image of the saliva components. As a whole, we have identified 299 non-redundant proteins in the *O. moubata* saliva and quantified the expression of 165 of them in both male and female saliva, among which 13 were significantly overexpressed in females and 40 in males. These results evidence important quantitative differences between sexes in the saliva proteome.

Conclusions

This work expand our knowledge of the *O. moubata* sialome, particularly of female ticks, by increasing the identification of novel salivary proteins and functions at the tick–host feeding interface. The integration of this new knowledge together with the information from the *O. moubata* sialotranscriptome will allow a more rational selection of the salivary candidates as antigen targets for tick vaccine development.

Background

Ticks are important blood-sucking arthropods in human and veterinary medicine due to their capacity to transmit a wide variety of infectious agents including viruses, bacteria and protozoans, which can cause severe diseases to people and pets as well as domestic and wild animals [1, 2].

*Ornithodoros moubata* is an argasid tick broadly distributed in numerous countries of eastern, central and southern mainland Africa and Madagascar. This tick lives in sylvatic environments associated mainly with warthogs and other animals inhabiting burrows, but it also invades synanthropic environments and colonises human dwellings and domestic animal premises, particularly pig premises [3]. In these countries, *O. moubata* has a significant medical and veterinary impact due to its function as a vector for *Borrelia duttoni*, a spirochete that causes tick-borne human relapsing fever.
(TBRF), and for the African swine fever (ASF) virus. The TBRF caused by *B. duttoni* is endemic in extensive zones of east Africa, affecting up to 6.4% of the population and causing perinatal mortality rates as high as 436 per 1000 [4, 5]. ASF is an acute febrile haemorrhagic disease of swine, with lethality rates close to 100%, which limits pig production and causes enormous economic losses in affected countries [6, 7].

In this context, elimination of domestic and peridomestic *O. moubata* populations would positively affect the prevention and control of these two severe diseases. The main strategy of tick control still is the application of chemical acaricides, despite their use having serious drawbacks such as the selection of tick resistant strains and toxicity as well as environmental and animal product contamination [8, 9]. In addition, chemical agents are ineffective for eliminating *O. moubata* from inside human dwellings and animal pens simply because they do not penetrate enough to reach the ticks in all the holes, cracks and fissures where they can take refuge, as was observed in Spain with *Ornithodoros erraticus* [10].

The development of anti-tick vaccines has proved to be an alternative eco-friendly and sustainable method for tick control with clear advantages over the application of chemical agents [11]. With that aim, years ago our team initiated the development of a vaccine for the control of *O. moubata* and focused our interest on the parasite antigens that are part of the tick-host interface and participate in the tick-host interplay, namely the salivary and intestinal tick antigens [12].

In the last decade, omics technologies such as next-generation sequencing (NGS) and high-throughput proteome analysis have been used to explore the sialomes and mialomes of several hard and soft tick species [13–15]. These studies have resulted in the identification of a range of tick molecules involved in the molecular mechanisms beneath tick haematophagy, tick-host interactions and pathogen transmission [13].

In *O. moubata*, we have recently applied omics technologies to analyse and characterise the transcriptome and proteome of the female tick midgut (mialome) before and after tick feeding [16, 17]. The obtained omics datasets have allowed us the selection of several vaccine candidate antigens, which were produced as recombinant proteins or as synthetic peptides and tested for vaccine efficacy in animal immunisation trials. Some of these candidates proved protective against *Ornithodoros* spp. and thus potentially useful to be included in vaccine formulations for tick control [18].

Regarding *O. moubata* salivary antigens, we have recently tackled their characterisation in depth by obtaining and analysing the female salivary transcriptome throughout the trophogonic cycle [19]. The newly obtained data have significantly increased the repertory of argasid salivary protein-coding sequences available in public databases and its analysis will facilitate the identification of new antigen candidates for the development of tick vaccines. Moreover, this annotated sialotranscriptome constitutes an invaluable reference database for future studies on the *O. moubata* salivary proteome, and it may be useful to confirm and expand former data on the salivary proteome of *O. moubata*, which were obtained in two former studies [20, 21]. These studies are the only ones on the *O. moubata* sialoproteome performed until now, and their results in terms of protein identification were limited by the scarcity of known tick sequences available at the time when these studies were executed.

The study by Oleaga et al. [20] analysed the proteome of the salivary glands of females by two-dimensional SDS-PAGE and MALDI-TOF MS, while the study by Diaz-Martín et al. [21] used LC-MS/MS to study the proteome of the female and male saliva separately.

Diaz-Martín et al. [21] observed noteworthy differences in the proteome composition between the sexes, as well as a high overrepresentation of some lipocalin proteins in female saliva, which hindered the identification of the most of the female saliva proteome, constituted by much less abundant proteins. Although equalising the saliva samples significantly increased the number of identified proteins, this treatment precluded the quantification of each protein component in the native saliva samples [22].
The specific aim of the present work is to expand the data on the salivary proteome of *O. moubata* adult ticks, particularly of female ticks, by solving the aforementioned drawbacks, as part of the in-depth characterisation and analysis of the *O. moubata* sialome. Knowing this sialome will allow identifying and selecting novel salivary antigens as targets for tick vaccines, and their subsequent testing in animal immunisation trials. We pay special attention to female ticks because they are a key developmental stage in the tick life cycle and vaccines may exert on them double deleterious effects, for instance, an increase in mortality and a reduction/inhibition of reproductive performance.

With that aim, in the current work, we have analysed samples of female and male saliva separately using two different mass spectrometry approaches: data-dependent acquisition (DDA) LC-MS/MS and sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS). This last technique is a specific variant of data-independent acquisition (DIA) methods and is an emergent technology that combines deep proteome coverage with quantitative consistency and precision [23].

To maximise the number of protein identifications, we have applied a proteomics informed by transcriptomics (PIT) analysis, which consisted of using the *O. moubata* salivary transcriptomic dataset previously obtained by RNAseq [19] as a reference database for protein identification.

Additionally, besides the analysis of the acquired proteomics data for protein identification and comparative quantification between female and male ticks, a comparative analysis of the performance and reproducibility between LC-MS/MS and SWATH-MS was done, which demonstrated greater usefulness and advantages of SWATH-MS over LC-MS/MS for the identification and quantification of the salivary proteins of *O. moubata* females.

### Material And Methods

#### Tick specimens

The *O. moubata* specimens used in the present work come from the IRNASA colony, which was established from specimens donated by Dr Philip Wilkinson (Institute for Animal Health, Pirbright, United Kingdom) captured in Malawi. This colony is kept at 28 °C, 85% relative humidity and 12/12 hours of light/dark, and is regularly fed on rabbits.

#### Tick saliva collection

Saliva was collected separately from each sex from newly moulted 4-month-old female and male ticks after stimulation of secretion with pilocarpine following the protocol described by Baranda et al. [24] with the following modifications.

Tick specimens were sequentially washed by immersion and shaking inside a 50 ml disposable centrifuge tube in the following series of 25-ml solutions: tap water, 3% hydrogen peroxide, two washes in distilled water, 70% ethanol and two more washes in distilled water. After that, ticks were dried on paper towels and immobilised with double side adhesive tape on a glass plate. Each tick was administered 1 µl of 1% pilocarpine hydrochloride (Sigma) in phosphate-buffered saline (PBS) pH 7.4 through the genital pore using a 5 µl Hamilton syringe, 33 gauge, 25 mm length needle. Shortly after stimulation, the tick started to move the chelicerae and emit small droplets of clear viscous saliva (< 0.5 µl), which were harvested from the tick mouthparts using a micropipette, and deposited on 150 μl of ice-cooled PBS. Saliva was collected from the ticks while perceptible emission continued, usually during 30–40 min after stimulation.

Three replicated biological saliva samples from each sex were prepared, each containing the secretion of 20 female ticks/sample (F1, F2, F3) or 40 male ticks/sample (M1, M2, M3). Saliva samples were centrifuged 10 min at 12,000 × g and 4 °C, and the supernatants were recovered and stored at -20 °C. Protein concentration was assessed by measuring the
absorbance at 280 nm in the NanoDrop 2000 spectrophotometer (ThermoFisher), and sample reproducibility was checked by SDS-PAGE (Additional file 1: Fig 1).

Protein digestion and sample preparation

Trypsin digestion and proteomic analyses were carried out at the SCSIE University of Valencia Proteomics Unit, a member of ISCIII ProteoRed Proteomics Platform.

Salivary proteins in each female (F1, F2, F3) and male (M1, M2, M3) saliva sample were analysed using an in-solution digestion method. For this, 20 µg of protein per sample were digested with Sequencing Grade Trypsin (Promega) as follows. First, protein samples were reduced using 10 mM dithiothreitol (DTT) (Sigma) to a final volume of 100 µl and the mixture incubated for 20 min at 60 °C. Then, proteins were alkylated with 5.5 mM iodoacetamide (IAM) (Sigma) to a final volume of 110 µl and incubated at room temperature for 30 min in the dark. Finally, each sample was digested with 800 ng of trypsin in a final volume of 118 µl and incubated overnight at 37 °C. The digestion was stopped with 12 µL of 10 % trifluoroacetic acid (TFA) (Fisher Scientific) in water. The mixtures were dried in a rotatory evaporator and dissolved in a final volume of 40 µl. All the reagents were prepared in 50 mM ammonium bicarbonate (ABC) (Sigma).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and building a spectral library

The peptides recovered from the in-solution digestion processing were analysed in a microESI qQTOF mass spectrometer (6600plus TripleTOF, ABSCIEX) in a data-dependent acquisition (DDA) mode. Briefly, for LC-MS/MS analysis, 5 µl from each digested sample (6 samples; 3 from females and 3 from males) were individually loaded using a nanoLC 425 (Eksigent) onto a trap column (3µ C18-CL 120 Å, 350 µm × 0.5mm; Eksigent) and desalted with 0.1% TFA at 5 µl/min for 5 min. Then, the peptides were separated using an analytical LC column (3µ C18-CL 120 Å, 0.075 × 150 mm; Eksigent) equilibrated in 5% acetonitrile (ACN) (Fisher Scientific) 0.1% formic acid (FA) (Fisher Scientific). Peptide elution was carried out with a linear gradient of 7–40% of buffer B in A for 45 min (A: 0.1% FA in water; B: 0.1% FA in ACN) at a flow rate of 300 nl/min. The eluted peptides were ionised in a source type Optiow applying 3.0 kV to the spray emitted, and the tripleTOF was operated in a data depending acquisition (DDA) mode. Full profile MS scans were acquired in the mass range of m/z 350–1400 for 250 ms in positive ion mode. The top 100 most intense ions were selected for fragmentation and MS/MS scans were acquired in the mass range of m/z 100–1500 for 25 ms in “high sensitivity” mode. Switch criteria used were the following: charge of 2+ to 4+, minimum intensity, and 100 counts per second. As is described below, the results of these analyses were used in a comparative analysis of the reproducibility and performance between LC-MS/MS in DDA mode and SWATH-MS.

For building a spectral library for SWATH-MS analysis, 2 µl of every digested sample were pooled and 5 µl of the pool were processed and analysed by LC-MS/MS exactly the same as described before for the individual samples. All the spectra obtained were combined and used for the generation of the reference spectral ion library as part of SWATH-MS analysis. The scheme of the workflow for spectral library creation as well as for sample processing is represented in Fig. 1.

Sequential window acquisition of all theoretical fragment ion spectra (SWATH) mass spectrometry (MS) analysis

SWATH-MS data for both female and male saliva samples were acquired on the same MS instrument used for LC-MS/MS.
The liquid chromatography conditions were the following. Five µl of each digested sample were loaded onto a trap column (LC Column, 12 nm, 3 µ Triart-C18, 0.5 × 5.0 mm; YMC) and desalted with 0.1% TFA at 10 µl/min for 5 min. The peptides were then loaded onto an analytical column (LC Column, Luna Omega 3 µm Polar C18, 150 × 0.3 mm, Capillary Phenomenex) equilibrated in 3% ACN 0.1% FA. Peptide elution was performed with a linear gradient of 3-35% B in A for 45 min (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nl/min.

The samples were acquired in a random order to avoid bias in the analysis. Sample was ionized in a source type Optiflow 1-50 µL micro, applying 4.5 kV to the spray emitter and the analysis was carried out in a data-independent acquisition (DIA) mode. Survey MS1 scans were acquired from 400–1250 m/z for 250 ms and 100 variable windows from 400 to 1250 m/z were acquired throughout the experiment. The total cycle time was 2.79 secs. The quadrupole resolution was set to ‘UNIT’ for MS2 experiments, which were acquired from 100–1500 m/z for 25 ms in ‘high sensitivity’ mode.

Identification and quantification of proteins

After LC-MS/MS, the wiff data files were processed using ProteinPilot v5.0 search engine (AB SCIEX). The Paragon algorithm [25] of ProteinPilot was used to search against a recently published protein fasta database derived from the O. moubata sialotranscriptome [19], (BioProject PRJNA667315). Searches were done with trypsin specificity, cys-alkylation and the search effort set to rapid.

To avoid using the same spectral evidence in more than one protein, the identified proteins were grouped based on MS/MS spectra by the Protein-Pilot Pro Group™ Algorithm, regardless of the peptide sequence assigned. The protein within each group that could explain more spectral data with confidence was depicted as the primary protein of the group.

Among the proteins identified by LC-MS/MS in DDA mode in each saliva sample, only those showing a Protein Pilot unused score above 1.3 (> 95% confidence) and a False Discovery Rate (FDR) lower than 1% were considered significant and included in the ensuing analyses. After manually inspecting all the proteins identified by LC-MS/MS, redundant identifications were removed by selecting the proteins with the highest score, and the hits of non-annotated transcripts were removed as well.

The identified proteins by SWATH-MS were quantified using PeakView 2.2 software from normalised label-free quantification intensity data. The generated spectral library was used as a database in Peak View 2.2 software for SWATH analysis and peaks from SWATH runs were extracted with a peptide confidence threshold of 95% and FDR lower than 1%. It was not set up to a minimum number of peptides for the identification. Quantitated proteins areas were normalised by total areas sum for differential expression analysis. In case of redundant identifications, the hit with a higher p-value was selected as representative and the normalised area values of the redundant proteins were added and the fold change recalculated.

Bioinformatic analyses

For functional annotations of the proteins identified, Uniprot IDs of the proteins were used to extract the Gene Ontology (GO) terms for biological process, molecular function, cellular component as well as cross-references in the InterPro, Pfam and Panther databases. The identified proteins were then functionally classified according to GO terms and bibliographic information, taking as a model the classification applied by Kim et al. [26] in their study of the proteome of Amblyomma americanum tick saliva.

Statistical analysis
The quantitative data obtained by PeakView were analysed using MarkerView (v1.2, AB SCIEX). First, areas were normalised by total areas sum. Multiexperiment Viewer (MeV) (http://www.tm4.org/mev.html) was used to identify the protein differentially expressed in saliva between female and male ticks using Welch’s t-test subjected to Bonferroni correction. Salivary proteins showing an adjusted p-value ≤ 0.05 were considered significantly differentially expressed between female and male ticks. The mean quantity of proteins in each saliva sample and the fold-change (ratio between the mean of proteins of female versus proteins of male) were calculated. The results of the hierarchical clustering analysis of the differentially expressed proteome profile of female and male samples were shown using a heat map after z-score normalisation using Euclidean distances.

Results And Discussion

Spectral library

Spectral libraries are essential for effective post-acquisition processing of SWATH data because they contain spectrometric data for all peptide precursors and their respective ion fragments, which are extracted from prior DDA MS experiments [27].

In the current work, we have generated a spectral library for O. moubata saliva proteins from DDA MS experiments involving 3 female and 3 male saliva samples. Additional file 2: Table S1 is the Protein Pilot report showing the spectrometric, statistic and identification data in the spectral library generated from O. moubata saliva proteins. This library included 5,497 spectra associated with 99% confidence, corresponding to 3,734 distinct peptides and 388 protein sequences with a FDR ≤ 1%. After eliminating, from the 388-protein list, up to 65 redundant identifications and 64 hits to non-annotated sequences from the O. moubata sialotranscriptome database, we obtained a final list of 259 non-redundant salivary proteins (Additional file 3: Table S2). This list includes all the proteins identified in the saliva of both sexes and was used as our reference library for the analysis of SWATH data.

Functional classification of these 259 proteins showed that the most numerous protein functional groups and families were the proteins involved in metabolic processes (n= 48), proteases (n= 28), antioxidants (n= 20), protease inhibitors (n= 17), and proteins with unknown function (n= 36) (Table 1). Typically, these families and groups are also the most abundantly represented in the sialomes of the soft and hard tick species analysed to date [15].

Results obtained by LC-MS/MS in DDA mode

Besides generating the spectral library, we have analysed individually each sample of female and male saliva by LC-MS/MS. For simplicity, the lists of protein identifications obtained in the analyses of the individual replicated samples were combined in two unique lists, one for each sex, which were later filtered by eliminating the redundancies and the non-annotated hits. Table 2 and Additional file 3: Table S2 show 195 and 64 proteins identified in male and female saliva, respectively. Of them, 36 proteins are shared by both sexes, and 159 and 28 are unique to males and females, respectively (Fig. 2).

The presence of some differences in protein composition between the saliva of O. moubata females and males can also be observed in the different protein band patterns shown by the saliva of each sex in SDS-PAGE (Additional file: Fig. S1), and it has already been described by Díaz-Martín et al. [21] who also confirmed the massive presence of lipocalin proteins in female saliva. The hyper-abundance of lipocalins would have hampered the detection of the less abundant proteins, which most likely would have remained below the detection limits of the assays. As proof of this was the fact that when the saliva samples were equalised and the “excess” of the hyperabundant proteins removed, the number of proteins identified in females increased very significantly [21, 28]. At that time, the work by Díaz-Martín et al. [21] provided
interesting novel information on the saliva proteome of males and females and revealed for the first time for soft tick differences in the saliva composition between both sexes. However, this study had an inconvenient, the method applied to increase the number of identifications, namely, the protein equalisation with the ProteoMiner Kit (BioRad), precluded the quantification of its components.

In ixodid ticks, the observed differences between males and females in the saliva composition are not surprising since both the feeding behaviour and the anatomy and functions of the salivary glands of both sexes are different [29,30]. However, the differences reported between *O. moubata* males and females in the saliva composition were somewhat surprising since typically soft tick adults, and specifically *O. moubata* adults, do not show anatomical differences in their salivary glands and they feed similarly. They ingest equivalent amounts of blood relative to their body weight and do it for a similar time, about an hour. This means that both sexes are obliged to overcome the same barriers and host defensive responses to complete blood ingestion, so it would be expected that both sexes would use the same repertory of anti-defensive salivary proteins [21].

In the current study, the different set of proteins identified by LC-MS/MS in female and male saliva also suggest qualitative differences in the salivary composition between sexes, following that was reported [21]. However, it must be noted that the reference database used in the present study for protein identification, i.e. the *O. moubata* sialotranscriptome, was obtained from female salivary glands only. Consequently, it might be assumed (i) that most likely the majority of the proteins identified in the present study, including those found in males only, would also be present in female saliva, and (ii) that the differences observed between sexes may be due more probably to quantitative differences in expression than to real absence/presence of particular proteins in one or another sex. As we will see later, the results of SWATH-MS also lend support to this idea.

Accordingly, it can be assumed that part of the qualitative differences observed by Diaz-Martin et al. [21] between the proteomes of male and female saliva, in which only 5.2% of the identified proteins were common, would possibly be due to quantitative differences.

**Comparing LC-MS/MS and SWATH-MS analyses**

To increase the number of protein identifications in both female and male saliva and quantify the protein expression level we analysed the saliva samples by two methods: LC-MS/MS operated in DDA mode and the free-label quantitative method SWATH-MS that operates in DIA mode.

Mass spectrometry methods that operate in DDA mode are based on the random selection and fragmentation of a fixed number of peptide precursors, generally the most intense peptide ions. On the other hand, in a SWATH-MS capture, all ionised peptides of a given sample that fall within a specified mass range are fragmented in a systematic and unbiased fashion using rather large precursor isolation windows [23]. Several published SWATH studies have demonstrated that SWATH-MS increases the sensitivity and the reproducibility of protein and peptide identification across multiple replicates [31,32]. Therefore, SWATH-MS might identify and quantify a higher number of proteins expressed simultaneously in *O. moubata* male and female saliva than LC-MS/MS, which in turn allows for comparing protein expression levels between both sexes.

Accordingly, we first assessed the performance and reproducibility of both methods in the identification of the *O. moubata* salivary proteome by comparing the results obtained by LC-MS/MS and SWATH-MS for the three replicate samples of each sex.

Regarding performance, LC-MS/MS analysis of the female samples resulted in the identification of 64 salivary proteins, while SWATH-MS of these same samples identified up to 165 salivary proteins (Table 2, Additional file 3: Table S2). Up to
40 of these proteins were identified by both methods, 24 exclusively by LC-MS/MS and 125 exclusively by SWATH (Fig. 3A). In male saliva, 195 and 165 proteins were identified by LC-MS/MS and SWATH-MS respectively (Table 2, Additional file 3: Table S2). Up to 136 male proteins were identified by both methods, 59 solely by LC-MS/MS and 29 solely by SWATH-MS (Fig. 3A).

With a 62% increase in the number of proteins identified, our data suggest that in the context of the female saliva, SWATH-MS is superior to DDA mode. However, in the male saliva DDA identified 15% more proteins than SWATH-MS. These data indicate that the benefit of SWATH over DDA MS, in the number of identified proteins, is unique for saliva of females and supports the notion that the performance of these techniques may be dependent on the fluid or the tissue analysed, as has been recently observed [32].

To assess the reproducibility in protein identification of both methods, we compared the data from the three biological replicates of female (F1, F2, F3) and male (M1, M2, M3) saliva. Fig. 3B shows that the reproducibility of LC-MS/MS was 27.3% and 36.6% in female and male saliva, respectively. On the other hand, the SWATH-MS reproducibility reached almost 100% (97-99%) in both sexes (Fig. 3C). Therefore, SWATH-MS outperformed DDA in the reproducibility of proteins identified across all three technical replicate analyses, and these results are in good agreement with previous reports [31].

Additional file 3: Table S2 shows the global results from both MS methods, which jointly identified 299 salivary proteins using the sialotranscriptome of O. moubata females as a reference database [19].

Quantification of the proteins identified in the female and male saliva by SWATH-MS

As already noted, SWATH-MS is a type of DIA method of analysis used to evaluate quantitatively complex samples with high reproducibility [33].

Using this technique, we have identified and quantified 165 proteins in the saliva of both female and male ticks, which were later classified in 21 groups and families (Table 2, Additional file 4: Table S3). Not unexpectedly, these groups/families coincide with the groups/families more abundantly represented in the O. moubata sialotranscriptome [19]. The groups with the highest numbers of proteins were proteins involved in metabolic processes (n = 30) and protein modification (n = 12), proteases (n = 19), lipocalins (n = 11), antioxidants (n = 10), regulation (n = 10), and unknown function (n = 24).

Fig. 4 represents the expression levels of the protein groups/families in both sexes, calculated as the mean spectral signal peak area for female and male saliva (Additional file 4: Table S3). The 24 proteins with unknown function have been excluded from the pie charts, and the groups containing 5 or fewer proteins (proteins involved in metabolism, signal transduction, protein synthesis, extracellular matrix, proteasome machinery and transporters) have been merged in one group named “other”.

Female saliva is predominantly composed of lipocalins as this group of proteins constitutes 95% of the saliva protein mass (Fig. 4A). The group includes 11 lipocalins, being moubatin (Q04669) and the so-named salivary lipocalin TSGP1 (F6K8G8) the most abundant as they respectively account for 40.39% and 55.33% of the total protein mass of this group (Additional file 4: Table S3).

Lipocalins are a large multigene protein family having dual functions as histamine and serotonin scavengers and as modulators of vertebrate inflammation and immunity [15]. Moubatin belongs to a lipocalin clade that includes proteins that inhibit platelet and neutrophil aggregation by scavenging of thromboxane A2 (TXA2) and proteins that inhibit complement activation by sequestering the C5 component [34]. TSGP1 belongs to the serotonin and histamine-binding
group of the soft tick lipocalins [35]. The current results confirm the previous report by Oleaga et al. [20] regarding the great abundance and numerous isoforms of TSGP1 discovered in the proteome of the salivary glands of *O. moubata* and support the notion that in *O. moubata* TSGP1 would be the main scavenger of histamine and serotonin. These pro-inflammatory biogenic amines accumulate at the tick-feeding site and need to be efficiently removed for the tick to successfully feed [33]. Regardless of its function, TSGP1 is highly immunogenic and strongly recognised by the serum from pigs bitten by *O. moubata*, which has made it a useful tool for serological diagnosis of parasitism by *O. moubata* [36]. In fact, a recombinant form of TSGP1 has been already used in several seroepidemiological studies of pig exposure to this tick in Madagascar, Mozambique and Nigeria [37-39].

The remaining non-lipocalin proteins represent only 5% of the total protein content of female saliva. Among them, proteases, protease inhibitors, antioxidants, and proteins involved in the metabolism of carbohydrates and lipids are the following more abundant groups representing between 0.82 and 0.42% of saliva protein content (Fig. 4A). In these groups, the more abundant proteins were a carboxipeptidase (B7QF76), a metalloprotease (Q09JT3), SCO-spondin-like (XP_021004313), enolase (D4P967), aldehyde dehydrogenase (B7QAL5), catalase (A0A2U8T6B2) and phospholipase A2 (M9W8K4) (Additional file 4: Table S3).

For several of these proteins, classified as housekeeping proteins, it is well established that they can also play important extracellular functions at the host-parasite interface, helping ticks to feed [40, 41]. For instance, in *O. moubata*, salivary enolase acts as a pro-fibrinolytic plasminogen activator receptor [40] and salivary secreted phospholipase A2 plays as an antagonist ligand of host P-selectin preventing P-selectin mediated endothelial activation [43].

In quantitative terms, the proteome of male saliva is remarkably different from that of female saliva (Fig. 4B). In male saliva, lipocalins are also the most abundant proteins -though they only account for 27.12% of the saliva protein content- and TSGP1 (F6K8G8) is also the most abundant individual lipocalin accounting the 60% of the protein content of this group (Additional file 4: Table S3). The following more abundant groups were protease inhibitors (17.55%) and the proteins involved in carbohydrate metabolism (15.71%) (Fig. 3B), with serpin-2 (Q06B74) (52.27%) and enolase (D4P967) (55.72%) being their most abundant individual components, respectively.

Serpin 2 is a well-characterised serine protease inhibitor that inhibits trypsin and thrombin, and interferes with platelet aggregation and blood clotting [44,45]. This activity and the above-referred pro-fibrinolytic activity of enolase [42] would collaborate in maintaining host blood fluidity helping tick to feed.

Among the 165 proteins quantified by SWATH-MS in the saliva from males and females, 53 were differentially expressed (p < 0.05) between the sexes (Table 3); 13 proteins were over-expressed in females and 40 were overexpressed in males. The signal peak areas of the differentially expressed proteins in each of the samples analysed were shown using a heat map after z-score normalisation, using Euclidean distances. The hit map shows two main clusters comprising the F1–F3 samples and M1–M3 samples, which correspond to the saliva of females and males respectively (Additional file 5: Fig. S2).

Fig. 5 represents the top 10 proteins that are differentially (p < 0.05) overexpressed in the saliva of female or male ticks. As expected according to the above-reported results, the top 10 overexpressed proteins in females were 5 lipocalins, including moubatin and TSGP1, phospholipase A2, apyrase, a metalloprotease, a salivary secreted basic tail protein and a salivary basic tailless protein (Table 3). Apyrase has been identified in the saliva of most hematophagous vectors including soft and hard ticks. It is an enzyme that hydrolyses ATP and ADP to AMP and prevents platelet and neutrophil aggregation and thrombus formation, facilitating blood feeding [46,47]. A recombinant form of the salivary apyrase of *O. moubata* induced protective, strong humoral responses in animal vaccine trials that reduced tick feeding and survival [46].

In *O. moubata*, as in other ixodid and argasid tick species, metalloproteases are one of the enzyme classes most abundantly represented in the saliva [14,19,48, 49]. In this study, LC-MS/MS and SWATH-MS identified 30 proteases, 16 of
them metalloproteases and 5 differentially expressed (p < 0.05) between females and males (Table 3).

Concerning basic tail and tailless proteins, their finding among the overexpressed proteins is not unexpected, as they are protein families abundantly found in the sialotranscriptomes of ixodid and argasid ticks, which suggest that they would play important and specific roles at the tick-host feeding interface [14,19,48, 49].

On the other hand, the top 10 proteins overexpressed in male saliva were two superoxide dismutases, two metabolic enzymes (adenosine amidase, hydroxypyruvate reductase), two proteins involved in immune mechanisms (gamma-interferon inducible lysosomal thiol reductase, spätzle alternatively spliced isoform), three proteins with unknown function, and ixodidin (Table 3 and Fig. 5). This last protein is an inhibitor of serine proteinases that shows antimicrobial activity [50].

As a whole, these results show that at least 165 out of 299 of the salivary proteins identified in the current study are shared by both sexes, which significantly reduces the range of qualitative differences between male and female saliva observed in previous works, where only 5.2% of the identified proteins were found in both sexes [21]. However, these results keep showing remarkable differences in the ratios of salivary proteins that males and females secrete in their saliva, which raises the question of the biological significance of these differences. It could be speculated that it may be related to the post-feeding blood processing or attraction and mating [51], but neither our current results nor a revision of the literature offered evidence to support or rule out such a notion and shed light on this matter.

Conclusions

Previous studies on the salivary transcriptome of *O. moubata* adults were handicapped by the hyper-abundance of lipocalins, especially in female saliva, that impeded the identification of the less abundant proteins. Equalisation of saliva samples increased protein identification but precluded quantification of its protein components [28, 21].

To overcome these hindrances, herein we have analysed the proteome of the saliva of *O. moubata* males and females via two mass spectrometry techniques, conventional LC-MS/MS and quantitative label-free SWATH-MS, and have implemented proteomics informed by transcriptomics (PIT) analysis using the recently obtained sialotranscriptome of *O. moubata* females as a reference database for protein identification [19].

The SWATH-MS proved superior to LC-MS/MS in the study of female saliva since it increased by 60% the number of identified proteins, enhanced the reproducibility of the results and provided a quantitative image of the saliva components. Additionally, the PIT analysis demonstrated its usefulness for proteomics studies of *O. moubata*, a non-model organism for which there are no genomic sequences available. PIT is being increasingly and successfully implemented in proteomics studies of tick saliva fuelled by the increasing number of tick sialotranscriptomes available resulting from the progressively more frequent application of NGS techniques to tick salivary glands [16, 30].

As a whole, we have identified 299 non-redundant proteins in the *O. moubata* saliva and quantified the expression of 165 of them in both male and female saliva, among which 13 were significantly overexpressed in females and 40 in males. These results evidence important quantitative differences between sexes in the saliva proteome and confirm in part the results obtained in earlier works using different methodological approaches.

These findings expand our knowledge of the *O. moubata* sialome, particularly of female ticks, by increasing the identification of novel salivary proteins and functions at the tick–host feeding interface.

The integration of this new knowledge together with the information from the *O. moubata* sialotranscriptome will allow a more rational selection of the salivary candidates as antigen targets for tick vaccine development and testing in animal immunisation tests. For example, hyperabundant proteins, such as lipocalins, should not be considered as vaccine targets.
because their massive presence in saliva would most likely preclude their complete neutralisation by the vaccine-induced antibodies.

Finally, we believe that testing multiantigenic vaccine formulations that include protective intestinal and salivary antigens should be encouraged since these formulations will target different biological processes and may provide synergic protective effects leading to more effective vaccines for the control of the *O. moubata* infestations and pathogen transmission.

**Abbreviations**

**ABC**
Ammonium bicarbonate; **AM:**Iodoacetamide; **DDA:**Data-dependent acquisition; **DIA:**Data-independent acquisition; **FDR:**False discovery rate; **DTT:**Dithiothreitol; **GO:**Gene Ontology; **LC-MS/MS:**Liquid chromatography-tandem mass spectrometry; **SWATH-MS:**Sequential window acquisition of all theoretical fragment ion spectra mass spectrometry; **TFA:**Trifluoroacetic acid.

**Declarations**

**Availability of data and materials**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [52] partner repository with the dataset identifiers PXD025657, PXD025658, PXD025660, PXD025680.

**Ethics approval**

Animal experimentation in this study was performed according to the regulations established by the Ethical and Animal Welfare Committee of the IRNASA, CSIC, Spain, and the corresponding EU Law (Directive 2010/63/EU).

**Competing Interests**

The authors declare that they have no competing interests.

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**Author contributions**

AO and RPS conceived and designed the study, interpreted the data and drafted the manuscript. ACM contributed to construction of tables and figures. MLV performed the proteomic analyses and provided critical review and revisions. All authors read and approved the final manuscript.

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Tables
Table 1
Number of salivary proteins in the spectral library. More details can be found in Additional file 3: Table 2.

| Classification                        | Number of proteins |
|---------------------------------------|--------------------|
| antioxidant/ detoxification           | 20                 |
| cytoskeletal                          | 14                 |
| extracellular matrix                  | 5                  |
| glycine rich                          | 8                  |
| heme/ iron binding                    | 2                  |
| immune related/defense                | 8                  |
| lipocalins                            | 15                 |
| metabolism, amino acids               | 2                  |
| metabolism, carbohydrate             | 13                 |
| metabolism, energy                   | 14                 |
| metabolism, lipids                    | 11                 |
| metabolism, nucleic acids             | 8                  |
| nuclear regulation                    | 7                  |
| protease                              | 28                 |
| protease inhibitor                    | 17                 |
| proteasome machinery                  | 6                  |
| protein modification                  | 14                 |
| protein synthesis                     | 1                  |
| regulation                            | 15                 |
| signal transduction                   | 6                  |
| transporter/ receptors                 | 8                  |
| transposable element                  | 1                  |
| unknown function                      | 36                 |
| **TOTAL**                             | **259**            |
Table 2
Number of identified proteins in saliva from female and male ticks by LC-MS/MS and SWATH-MS. More details can be found in Additional file 3: Table 2.

| Classification                  | LC-MS/MS male | LC-MS/MS female | SWATH-MS (male and females) |
|--------------------------------|---------------|----------------|----------------------------|
| antioxidant/detoxification     | 15            | 3              | 10                         |
| cytoskeletal                   | 10            | 1              | 9                          |
| extracellular matrix           | 4             | 2              | 4                          |
| glycine rich                   | 9             | 1              | 8                          |
| heme/iron binding              | 0             | 2              | 1                          |
| immune related/defense         | 8             | 2              | 6                          |
| lipocalins                     | 10            | 11             | 11                         |
| metabolism, amino acids        | 1             | 1              | 1                          |
| metabolism, carbohydrates      | 13            | 2              | 8                          |
| metabolism, energy             | 13            | 1              | 12                         |
| metabolism, lipids             | 10            | 2              | 6                          |
| metabolism, nucleic acids      | 7             | 3              | 3                          |
| nuclear regulation             | 0             | 0              | 1                          |
| protease                       | 18            | 12             | 19                         |
| protease inhibitor             | 15            | 5              | 9                          |
| proteasome machinery           | 3             | 0              | 2                          |
| protein modification            | 14            | 1              | 12                         |
| protein synthesis               | 1             | 0              | 1                          |
| regulation                     | 11            | 2              | 10                         |
| signal transduction            | 6             | 0              | 3                          |
| transporter/receptors           | 7             | 1              | 5                          |
| unknown function               | 20            | 12             | 24                         |
| TOTAL                          | 195           | 64             | 165                        |
Table 3
Proteins detected by SWATH-MS that are differentially expressed (p < 0.05) between female and male saliva. vs, versus.

| Classification               | Accession | Protein name                                           | Mean signal peak area (n = 3) | Fold Change (female vs male) | p-value   |
|------------------------------|-----------|--------------------------------------------------------|-----------------------------|------------------------------|-----------|
| antioxidant/detoxification   | A0A2U8T6B2 | Catalase                                               | 1.11                        | 4.83                         | 0.23      | 3.87E-02 |
|                              | Q09JE3    | Superoxide dismutase [Cu-Zn]                          | 0.39                        | 3.69                         | 0.10      | 1.26E-03 |
|                              | XP_026761353 | Superoxide dismutase [Cu-Zn]                        | 0.31                        | 4.81                         | 0.07      | 2.86E-02 |
|                              | A6N9S1    | Thioredoxin peroxidase                                 | 0.51                        | 3.24                         | 0.16      | 7.70E-04 |
| cytoskeletal                 | A4UTU3    | Beta-actin                                             | 5.51                        | 50.56                        | 0.11      | 4.02E-03 |
|                              | XP_023220065 | Moesin/ezrin/radixin homolog 1                     | 5.63                        | 26.56                        | 0.21      | 7.10E-04 |
|                              | F0JA36    | Profilin                                               | 3.05                        | 10.49                        | 0.29      | 5.96E-03 |
| extracellular matrix         | A0A3G1T1P7 | Mucin-like                                             | 2.31                        | 7.13                         | 0.32      | 6.90E-03 |
|                              | A0A3B0JRB0 | Peritrophin-1                                         | 1.22                        | 4.84                         | 0.25      | 1.67E-02 |
| glycine rich                 | XP_021703737 | Glycine-rich cell wall structural protein 1.8-like    | 0.90                        | 3.50                         | 0.26      | 9.07E-03 |
| immune related/defense       | B7Q4R4    | Double sized immunoglobulin G binding protein A       | 8.67                        | 48.30                        | 0.18      | 4.34E-02 |
|                              | B7QIC3    | Gamma-interferon inducible lysosomal thiol reductase   | 0.19                        | 3.71                         | 0.05      | 1.27E-03 |
|                              | B7QFC1    | Spatzle alternatively spliced isoform 11.27           | 4.22                        | 131.89                       | 0.03      | 2.34E-02 |
| lipocalin                    | Q04669    | Moubatin                                               | 4,964.65                    | 48.28                        | 102.84    | 2.82E-03 |
|                              | B2D2A7    | Salivary lipocalin                                     | 84.69                       | 19.96                        | 4.24      | 2.38E-02 |
|                              | B2D2D9    | Salivary lipocalin                                     | 3.98                        | 0.90                         | 4.40      | 9.64E-03 |
|                              | F6K8G8    | Salivary lipocalin (TSGP1)                             | 6,801.11                    | 694.65                       | 9.79      | 1.80E-04 |
|                              | A6N9Y0    | Salivary secreted protein                              | 23.36                       | 5.77                         | 4.05      | 6.85E-03 |
| metabolism                   | B7PV15    | Glyoxylate/hydroxypyruvate reductase                   | 2.58                        | 55.15                        | 0.05      | 4.88E-02 |
|                              | B7PLL4    | Fructose-bisphosphatase                                | 1.04                        | 2.04                         | 0.51      | 2.18E-02 |
| Classification          | Accession | Protein name                                      | Mean signal peak area (n = 3) | Fold Change (female vs male) | p-value  |
|-------------------------|-----------|--------------------------------------------------|------------------------------|------------------------------|----------|
|                         |           | female (*10^4) | male (*10^4) |                          |          |
| B7PFJ2                  | Isocitrate dehydrogenase [NADP] | 0.18 | 1.47 | 0.12 | 5.90E-04 |
| XP_013773334            | Lysosomal alpha-glucosidase-like | 8.50 | 12.39 | 0.69 | 4.10E-02 |
| XP_023224832            | Acyl-CoA-binding protein-like | 0.20 | 1.33 | 0.15 | 3.74E-03 |
| B7QMWO                  | Fatty acid-binding protein FABP | 8.94 | 46.22 | 0.19 | 3.48E-03 |
| M9W8K4                  | Phospholipase A2 | 38.00 | 5.98 | 6.36 | 3.39E-02 |
| B7PJJ3                  | Adenosine deaminase, putative | 7.85 | 89.73 | 0.09 | 1.57E-02 |
| M9WFX8                  | Apyrase | 24.37 | 7.79 | 3.13 | 2.96E-03 |
| protease                | E0AD92    | Angiotensin-converting enzyme | 8.46 | 44.25 | 0.19 | 1.00E-03 |
|                        | A0A087UKQ1 | Carboxypeptidase | 0.58 | 3.45 | 0.17 | 7.00E-04 |
|                        | B7QF76    | Carboxypeptidase | 33.37 | 38.82 | 0.86 | 1.41E-02 |
|                        | B7QBM2    | Dipeptidyl aminopeptidase III | 0.84 | 1.77 | 0.47 | 2.69E-02 |
|                        | A0A482VVE0 | Endothelin-converting enzyme 1 | 2.47 | 17.33 | 0.14 | 7.80E-04 |
|                        | Q09JT3    | Metalloprotease | 21.92 | 5.97 | 3.67 | 3.23E-02 |
|                        | Q1ZZW9    | Metalloproteinase | 3.20 | 1.12 | 2.85 | 3.82E-02 |
| protease inhibitor      | B2D258    | Ixodidin | 0.95 | 14.56 | 0.06 | 4.59E-02 |
| protein modification    | A0A1L5L8R3 | Heat shock protein 70-8235 | 0.33 | 1.53 | 0.21 | 2.05E-03 |
|                        | XP_023224182 | Heat shock protein 83-like | 3.81 | 11.84 | 0.32 | 7.58E-03 |
|                        | B7PAR6    | Heat shock protein, putative | 9.47 | 22.96 | 0.41 | 1.45E-02 |
|                        | Q86G69    | Heat shock-related protein | 0.61 | 3.82 | 0.16 | 3.61E-03 |
|                        | A0A0A0N845 | Small heat shock protein I | 15.03 | 47.30 | 0.32 | 3.04E-03 |
| Classification       | Accession   | Protein name                                           | Mean signal peak area (n = 3) | Fold Change (female vs male) | p-value  |
|----------------------|-------------|--------------------------------------------------------|------------------------------|-----------------------------|----------|
|                      |             |                                                        | female (×10^4)               | male (×10^4)                |          |
| protein synthesis    | B7Q349      | Elongation factor 1-alpha                              | 1.34                         | 5.84                        | 0.23     | 1.70E-04 |
| regulation           | XP_013771971| L-asparaginase-like isoform X1                         | 0.11                         | 0.78                        | 0.14     | 2.86E-02 |
|                      | XP_023224526| Protein/nucleic acid deglycase DJ-1-like               | 0.84                         | 4.39                        | 0.19     | 1.55E-02 |
| signal transduction  | A0A076FFP4  | Calmodulin                                             | 0.51                         | 2.68                        | 0.19     | 7.70E-05 |
| transporter/receptor | B7PIZ1      | Rab GDP dissociation inhibitor                         | 2.76                         | 8.05                        | 0.34     | 1.85E-02 |
| unknown function     | XP_023235971| Ectonucleotide pyrophosphatase/phosphodiesterase family member 1-like | 19.42                        | 7.65                        | 2.54     | 9.09E-05 |
|                      | F0J8E8      | Hypothetical secreted protein 1669                    | 10.87                        | 141.08                      | 0.08     | 2.03E-02 |
|                      | A6N9P6      | Salivary basic tailless protein                        | 3.39                         | 0.54                        | 6.30     | 3.91E-03 |
|                      | B2D272      | Salivary secreted basic tail protein                   | 21.65                        | 3.00                        | 7.21     | 8.54E-03 |
|                      | A0A3B0JWS0  | Uncharacterized protein                                | 0.55                         | 2.25                        | 0.24     | 4.83E-02 |
|                      | B7PLU7      | Uncharacterized protein                                | 0.34                         | 46.32                       | 0.01     | 4.69E-02 |
|                      | B7Q6K2      | Uncharacterized protein                                | 3.23                         | 1.09                        | 2.96     | 7.50E-03 |
|                      | B7QC55      | Uncharacterized protein                                | 14.66                        | 587.39                      | 0.02     | 6.23E-05 |

Figures
Figure 1

Schematic of experimental workflow employed in this study

Figure 2

Venn diagram depicting number and overlap of non-redundant salivary proteins detected by LC-MS/MS.
Figure 3

Comparative analysis of the performance of SWATH-MS and LC-MS/MS. A) Venn diagram depicting number and overlap of salivary proteins detected by LC-MS/MS and SWATH. B) and C) Venn diagrams depicting the number and overlap of unique proteins detected by LC-MS/MS and SWATH respectively across three biological replicates in female (F1, F2, F3) and male (M1, M2, M3) tick saliva.
Figure 4

Expression level of the identified proteins classified by functional groups. The expression level was calculated as the mean spectral signal peak area in female (F1, F2, F3) (A) and male (M1, M2, M3) (B) samples.
Figure 5

Top 10 proteins that are differentially (p < 0.05) overexpressed in female saliva (red) or male saliva (blue).

Supplementary Files

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