Edinburgh Research Explorer

Genome annotation improvements from cross-phyla proteogenomics and time-of-day differences in malaria mosquito proteins using untargeted quantitative proteomics

Citation for published version:
Imrie, L, Le Bihan, T, O'Toole, A, Hickner, PV, Dunn, WA, Weise, B & Rund, SSC 2019, 'Genome annotation improvements from cross-phyla proteogenomics and time-of-day differences in malaria mosquito proteins using untargeted quantitative proteomics', PLoS ONE, vol. 14, no. 7. https://doi.org/10.1371/journal.pone.0220225

Digital Object Identifier (DOI):
10.1371/journal.pone.0220225

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
PLoS ONE

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Genome annotation improvements from cross-phyla proteogenomics and time-of-day differences in malaria mosquito proteins using untargeted quantitative proteomics

Lisa Imrie1☯, Thierry Le Bihan1,2,3☯, Áine O’Toole4, Paul V. Hickner5, W. Augustine Dunn6, Benjamin Weise2, Samuel S. C. Rund2,5*  

1 SynthSys–Synthetic and Systems Biology, School of Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom, 2 Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh, United Kingdom, 3 Rapid Novor, Kitchener, Ontario, Canada, 4 Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, United Kingdom, 5 Eck Institute for Global Health, University of Notre Dame, Notre Dame, Indiana, United States of America, 6 Boston Children’s Hospital, Boston, Massachusetts, United States of America  

☯ These authors contributed equally to this work.  
* srund@nd.edu

Abstract

The malaria mosquito, Anopheles stephensi, and other mosquitoes modulate their biology to match the time-of-day. In the present work, we used a non-hypothesis driven approach (untargeted proteomics) to identify proteins in mosquito tissue, and then quantified the relative abundance of the identified proteins from An. stephensi bodies. Using these quantified protein levels, we then analyzed the data for proteins that were only detectable at certain times-of-the-day, highlighting the need to consider time-of-day in experimental design. Further, we extended our time-of-day analysis to look for proteins which cycle in a rhythmic 24-hour (“circadian”) manner, identifying 31 rhythmic proteins. Finally, to maximize the utility of our data, we performed a proteogenomic analysis to improve the genome annotation of An. stephensi.

We compare peptides that were detected using mass spectrometry but are ‘missing’ from the An. stephensi predicted proteome, to reference proteomes from 38 other primarily human disease vector species. We found 239 such peptide matches and reveal that genome annotation can be improved using proteogenomic analysis from taxonomically diverse reference proteomes. Examination of ‘missing’ peptides revealed reading frame errors, errors in gene-calling, overlapping gene models, and suspected gaps in the genome assembly.

Introduction

Anopheles stephensi is a major malaria vector in southern Asia where its geographic range extends across the Indian subcontinent [1]. Research on the African Anopheles gambiae mosquito has demonstrated that the behavior and physiology of the mosquito is highly dependent on circadian biology and time-of-day. For example, ~20% of An. gambiae genes were
Wellcome Trust (No. 095831) for the Centre for Immunity, Infection and Evolution. Áine O’Toole was funded by the Wellcome Trust (202769/Z/16/Z; PhD programme in Hosts, Pathogens and Global 348 422 Health). The LC-MS QExactive equipment was purchased by a Wellcome Trust Institutional Strategic Support Fund and a strategic award from the Wellcome Trust for the Centre for Immunity, Infection and Evolution (095831/Z/11/Z). Rapid Novor provided support in the form of a salary for author TLB, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The remaining funders also had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: TLB has received salary from Rapid Novor. TLB’s employment at Rapid Novor does not alter our adherence to PLOS ONE policies on sharing data and materials. The other authors have declared that no competing interests exist. The specific roles of all authors are articulated in the ‘author contributions’ section.

rhythmically expressed over the 24-hour day [2]; rhythmically expressed mosquito olfaction genes correspond with rhythmic proteins levels and time-of-day changes in electrophysiological sensitivity to host odorants [3]; and time-of-day effects are associated with mosquito insecticidal resistance[4]. An. stephensi has been demonstrated to have 24-hour nocturnal rhythms of flight behavior that persists even in the absence of light:dark cues [5]. Finally, rhythms in the biology of the mosquito, and indeed possibly in the human host and plasmodium parasite, may interact to affect disease transmission [6–8].

To date, the genomes of two strains of An. stephensi have been sequenced, one from India and one from Pakistan (SDA-500) [9, 10]. To our knowledge, proteomics in this species is limited to an Edman degradation of their salivary glands [11]; mass spectrometry proteomics analysis of salivary proteomes [11, 12]; fat bodies [13, 14]; midguts/fat bodies [14]; a mass spectrometry proteomics analysis of ageing in the head and thorax [15]; and a recent work across multiple tissues which included genome annotation improvements [16].

In An. gambiae, several mass spectrometry-based studies have been performed on various tissues, including the antennae, head, body, midgut peritrophic matrix, salivary glands, and cuticle [3, 17–20]. Proteomic experiments can be used to identify post-translational modification, improve genome annotation, and to identify and quantify proteins in a biological sample [16, 21, 22].

A previous study in An. gambiae mosquito antennae utilized targeted quantitative proteomics, in which the mass spectrometer was tuned to specifically identify and quantify the protein abundance of proteins from an a priori list of genes of interest [3] where only targeted proteins are interrogated. Targeted proteomics is a powerful technique, allowing the verification of a defined working hypothesis on specific proteins that are quantified.

In the present work, we used a non-hypothesis driven approach (untargeted proteomics) to identify proteins in mosquito tissue. In addition, we quantified the relative abundance of the identified proteins from An. stephensi bodies. Such an untargeted, label free, quantitative analysis has been used on diverse tissues such as mammalian cells, yeast, bacteria, and Ostreococcus tauri algae [23–26]. Using these quantified protein levels, we then analyzed the data for proteins that were only detectable at certain times-of-the-day, highlighting the need to consider time-of-day in experimental design. Further, we extended our time-of-day analysis to look for genes that are not only detectable at certain times-of-day, but which cycle in a rhythmic 24-hour (“circadian”) manner.

Annotation of the An. stephensi genome is far less complete than that of the model mosquito, An. gambiae. Proteogenomic analysis can be used to improve these annotations, particularly by experimentally validating computationally-derived open reading frame (ORF) predictions [27]. Additionally, proteogenomic analysis can be used in the identification of variant sequences and novel splicing sites [28, 29]. In these analyses, peptides detected using mass spectrometry were compared with reference proteomes. Peptide sequences not found in predicted protein coding regions indicate mis-annotations such as missing exons or entire missing genes, while peptide sequences with single amino-acid differences between the experimentally detected sequence and reference proteome may represent sequencing errors or polymorphisms. Here we apply proteogenomic analysis to An. stephensi, but employing a novel protocol, we reference over 30 predicted proteomes from other vector species to search against our experimentally derived peptides. We reveal that genome annotation can be improved using proteogenomic analysis from taxonomically diverse reference proteomes.
Results and discussion

Global survey of the proteome without fractionation

Using an untargeted proteomics approach without any fractionation on pooled samples of 10 whole mosquitoes harvested across the 24 hr day, we have identified 12641 unique peptides (having a Maxquant score of 45 and more) mapping to ~1700 (identified with at least 2 peptides) *An. stephensi* proteins (S1 Table). *An. stephensi* is thought to have ~11,789 genes [9] thus, with no fractionation we observed 13% of the predicted proteome. Future studies could increase the number of detectable proteins by fractionation or by using an iTRAQ or TMT labelling strategy combined with fractionation as a good compromise between high number of samples and fractionation.

Time-of-day dependent changes in detectable genes

In order to determine if there is a time-of-day dependent ability to detect proteins, mosquito bodies were collected every four hours from three staggered time courses (Fig 1) of between 28–44 hr (Fig 1). Note in this collection protocol, each of five times-of-day are sampled, independently, five times (see Fig 1). Untargeted, quantitative proteomics was performed (S2 Table, S3 Table), and a total of 1525 body proteins were deemed quantifiable (identified with at least two component peptides) from the body samples.

Our data revealed differences in the total number of proteins that were quantifiable at any given time-of-day (583–733 proteins per time-of-day) (Fig 2A). When the identity of proteins detectable (i.e. quantity > 0) is considered, there are a number of proteins only detectable at certain times-of-day. Whereas 489 proteins were detectable at all sampling times, there were between 45 and 72 proteins that were only detectable at 2–5 times-of-day, and 134 were only detectable at a single time-of-day (Fig 2B). Not surprisingly, proteins that had a higher average abundance were generally detectable at more times-of-day (Fig 2C).

Identification of rhythmic genes

We extended our analysis further by looking for genes that were rhythmically expressed using an algorithm (JTK_CYCLE) specifically designed for looking for “circadian” expression patterns. First, we analyzed the subset of 357 proteins that were detected at all time points, and where >1 peptide was used to identify each protein in each sample at each timepoint. Of these

![Fig 1. Sampling protocol for time-of-day assays.](https://doi.org/10.1371/journal.pone.0220225.g001)
proteins, ANOVA revealed 90 proteins, where at least one time point was significantly different from the others ($p < 0.1$). Next, from the list of proteins with statistically significant time-of-day differences in protein concentration (90 proteins), we proceeded to analyze those proteins for 24 hr daily rhythms in abundance (rather than only a simple time-of-day difference) using the JTK_CYCLE algorithm. This algorithm is used to mine ‘omic data for such 24 hour rhythms [30, 31], and we thus applied it to our data. JTK_CYCLE identified 31 proteins as having rhythmic expression (Fig 3). As *A. stephensi* has not been extensively annotated, we mapped these 31 proteins to their homologues in *Ae. aegypti*, *An. gambiae*, *Culex quinquefasciatus*, and/or *D. melanogaster* to assign a name/function to each protein (Table 1). We note these rhythmic proteins display a wide range of phases (times-of-day when proteins peak) and abundance amplitudes (Fig 3, Table 1).

**Conservation of rhythmicity across species.** We next searched published studies of rhythmic gene expression in *Ae. aegypti* [32], *An. gambiae* [2], and *D. melanogaster* [33] to determine if homologues of rhythmic *A. stephensi* protein were rhythmic in these species at the gene expression level. Indeed, we determined that of our 31 rhythmically identified *A. stephensi* proteins, 17 had homologues in at least one of the other three species with rhythmic expression of the same gene (Table 1). This represents ~55% of identified *A. stephensi* rhythmic proteins. For example, considering *A. stephensi* protein ASTEI01494, which is predicted to be glycerol 3-phosphate based on homology to *D. melanogaster* (FBgn0001128) and *An. gambiae* (AGAP007593), we find the *A. stephensi* protein abundance is rhythmic, as are the gene expression levels in *An. gambiae* gene expression and *D. melanogaster* expression levels. Similarly, protein abundance levels of ASTEI00584 and expression levels of the homologous hydrogenase genes in *Ae. aegypti* (AAEL010814)/*An. gambiae* (AGAP003167) are also both rhythmic (see Table 1).

**Proteogenomic analysis.** In order to determine if our proteomics work could be used to improve the *A. stephensi* genome, we next performed a proteogenomic analysis. Two sets of
computed proteomes from VectorBase were utilized: (1) the *An. stephensi* proteome (Indian strain peptide sequences, Astel2.3 geneset with 11,789 entries); and (2) the complete proteomes stored in VectorBase (here referred to as "All Vectors", with >566,000 entries). "All vectors" comprises 39 proteomes (S4 Table), including other mosquito proteomes and other
Table 1. Rhythmic proteins identified.

| Indian-strain geneID | SDA-500 geneID | JTK_CYCLE values | Dipteran homologues ** | Ensembl ID | Species: Name |
|----------------------|----------------|------------------|------------------------|------------|---------------|
| ASTEI0121           | ASTE003060     | 0.013 18         | CPIJ013361 CQUI: Tropomyosin-1 |
| ASTEI00584          | ASTE004515     | 0.018 20         | AAEI010814 AAEG: isocitrate dehydrogenase |
| ASTEI00675          | ASTE008899     | 0.015 8          | AGAP003167 AGAM: NAD(P) transhydrogenase |
| ASTEI00949          | ASTE002146     | 0.090 16         | AGAP003937* AGAM: AGAP003937 |
| ASTEI00949          | ASTE011453     | 0.016 20         | AGAP003936* AGAM: small nuclear ribonucleoprotein D2 |
| ASTEI01125          | ASTE005445     | 0.030 20         | CPIJ011528 CQUI: NADH dehydrogenase iron-sulfur protein 2, mitochondrial |
| ASTEI01494          | ASTE001799     | 0.012 22         | FBgn0019968 DMEL: Kinesin-73 |
| ASTEI01494          | ASTE008891     | 0.038 22         | FBgn0001128* DMEL: GPD-C |
| ASTE008307          | AASE003726     | 0.020 8          | AASE003211* AAEG: beta-carotene dioxygenase |
| ASTEI02163          | ASTE010238     | 0.063 20         | AGAP005558 AGAM: peptidase (mitochondrial processing) beta |
| ASTEI02221          | ASTE009536     | 0.026 20         | AGAP005627* AGAM: creatine kinase |
| ASTEI02598          | ASTE008307     | 0.056 18         | AGAP006936* AGAM: Mitochondrial cytochrome c1 heme protein |
| ASTEI02810          | ASTE010498     | 0.071 18         | CPIJ000098 CQUI: Electron transfer flavoprotein-ubiquinone oxidoreductase |
| ASTEI03561          | ASTE000276     | 0.001 10         | FBgn0265949 DMEL: RH35990p |
| ASTEI03834          | ASTE011334     | 0.056 4          | AAEI007698* AAEG: PIWI |
| ASTE003184          | AASE003184     | 0.001 14         | AGAP010051* AGAM: AGAP010051 |
| ASTEI04436          | AASE006286     | 0.090 18         | AGAP000720* AGAM: Neuronal cell adhesion molecule |
| ASTEI05481          | AASE011491     | 0.044 16         | CPIJ019398 CQUI: Myosin light chain 2 |
| ASTEI05637          | AASE010982     | 0.009 18         | AGAP011131* AGAM: F-type H+ transporting ATPase subunit d |
| ASTE00678           | ASTE02102      | 0.050 22         | AGAP012000 AGAM: 40S ribosomal protein 26 |
| ASTEI06447          | ASTE009294     | 0.003 2          | AASE003427* AAEG: 40S ribosomal protein 16 |
| ASTE06644           | ASTE008693     | 0.008 8          | AGAP008364* AGAM: thioester-containing protein 15 |
| ASTE06854           | ASTE001249     | 0.034 10         | FBgn0031021 DMEL: NADH dehydrogenase (ubiquinone) 18 kDa subunit |
| ASTE07075           | ASTE002586     | 0.026 12         | AGAP004055* AGAM: 2-oxoglutarate dehydrogenase E2 component |
| ASTE07469           | AASE01425      | 0.071 18         | AASE014913 AAEG: Pyruvate kinase |
| ASTEI05885          | AASE010928     | 0.071 10         | AGAP004146* AGAM: Ras-related protein Rab-1A |
| ASTE08990           | AASE000090     | 0.007 10         | AGAP010895* AGAM: spectrin beta |
| ASTE09101           | ASTE006195     | 0.003 18         | AGAP007841* AGAM: F-type H+ transporting ATPase subunit delta |
| ASTE09205           | ASTE008202     | 0.023 18         | AGAP011800 AGAM: Transaldolase |
| ASTE09484           | AASE004410     | 0.034 18         | AGAP010404* AGAM: Glutathione S-transferase |
| ASTE09679           | AASE002472     | 0.020 10         | AAEI007881* AAEG: AAEI007881 |
| ASTEI10862          | AASE001429     | 0.004 10         | FBgn0016693 DMEL: Putative Achaete Scute Target 1 |
| ASTEI11367          | No match       | 0.012 16         | AGAP007122 AGAM: Tubulin, alpha 1 |

* The listed homologue has been found to be expressed rhythmically in *Aedes aegypti* [32], *Anopheles gambiae* [2], or *Drosophila melanogaster* [33]
** Only rhythmic homologues and/or a representative named homologue in AAEG: *Ae. aegypti*, AGAM: *An. gambiae*, CQUI: *Culex quinquefasciatus*, or DMEL: *D. melanogaster* is provided.
*** The calculated time-of-day, in zeitgeber (ZT) time, when protein abundance peaks where ZT0 is lights on and ZT12 is lights off.

https://doi.org/10.1371/journal.pone.0220225.t001
vectors such as snails, ticks, and kissing bugs. In other words, we analyzed the spectra generated from each peptide and compared it against both a list of An. stephensi computed peptide spectra and our “All Vectors” computed peptide spectra. A spectrum that was found in “All Vectors” but not An. stephensi was deemed to be missing from the An. stephensi genome, since we had proteomic evidence of the existence of the peptide as computed from other species (of varying degrees of relatedness).

There were 57,726 peptide matches between our data and the An. stephensi (Indian strain) proteome in VectorBase. Using a score cut-off based on identity score (average of 35.6 for “All Vectors” and 19 for An. stephensi) the number of retained high confident peptides matches decreased to 82,402 and 32,949 for “All Vectors” and An. stephensi, respectively. We manually validated all hits which were found in both sets and removed any peptides that had identical or nearly identical scores in both “All Vectors” and An. stephensi (e.g. isoleucine to leucine permutations are isobaric and are thus not easily distinguishable by mass spectrometry).

From the preceding analyses and filtering (such as excluding matches to SDA-500, another An. stephensi strain) we identified a total of 792 (S5 Table) high-confidence matches between peptide sequences that are ‘missing’ from the An. stephensi (Indian strain) proteome, yet are found in the 39 “All Vectors” (S4 Table) proteomes. Some identified ‘missing’ peptides had only a match in another single organism, while other peptides could be found in up to 35 other proteomes. By also combining peptide sequences that are completely contained within longer peptide sequences that were also detected, we are left with 239 unique peptide groups (i.e. ATAQLIESIK, ATAQLIE, and ATAQL count as one peptide group) that were detected using proteomics in An. stephensi samples, that matched at least one peptide sequence in one of the 39 other proteomes, but are not found in the currently available predicted Indian strain An. stephensi proteome. Matches were found across a wide phylogenetic diversity, from lice to snails—not just in other mosquitos or diptera (Supplemental 5, Fig 4). These matches suggest possible genome annotation errors, which we next analyzed.

Identification of potential errors in the genome annotations. Further analysis of the peptides detected in our study, but missing from the An. stephensi proteome (Indian strain, Astel2.3), revealed potential errors in the genome annotations and/or assembly (S6 Table). Of the 239 peptides missing from the An. stephensi, but with matches found in “All Vectors,” were 2 peptides with 100% identity to a transcript based on tBLASTn analysis. These were found to be in a different reading frame than the annotated transcript and represent missing gene models where two genes overlap—a common phenomenon in eukaryotic organisms [34]. tBLASTn analysis revealed 25 peptides with 100% identity to a genome scaffold but not to a transcript. These are most likely genes that were not called by the gene prediction software and are missing from the current geneset (Astel2.3). There were 94 peptides that had high homology to a genomic region that contained a SNP or a mismatch causing a frameshift mutation, which could be either mutations or sequencing errors. Finally, 120 peptides were not found in the genome using standard BLAST tools and may represent gaps in the genome assembly.

Conclusions

In this work we performed untargeted quantitative proteomics on An. stephensi mosquito samples to answer three different questions: (1) Are there qualitative, time-of-day differences in the peptides? (2) What proteins can be detected as rhythmic in a 24 hour “circadian” manner? and (3) Can the An. stephensi genome be improved using proteomic data compared against genomes of other species?
By collecting mosquito samples every four hours across the day, we determined that there are time-of-day differences in the number and quantity of proteins that are detectable at any given time-of-day. Previous work in *An. gambiæ* revealed dusk (ZT12) was the time-of-day that had the greatest number of rhythmic genes which had their peak in expression [2]. It was hypothesized that this is due to the massive change in mosquito behavior and physiology as it goes from a resting state during the day to an active, host seeking mosquito at night [2]. Congruent with this, the greatest number of detectable proteins were detected at dusk in *An. stephensi*. Not surprisingly, proteins that had a higher protein abundance were detectable at more times-of-the day. A total of 134 proteins were detected only at one time-of-day, and 489 were detectable at all times-of-day.

We next extended our analysis from a question of detectability at different times-of-day, to see if 24-hour (“circadian”) levels of protein abundance could be detected in our dataset. Previous work in the *An. gambiæ* mosquito utilized targeted quantitative proteomics, whereby the protein abundance of proteins from an *a priori* list of genes of interest [3, 17] was quantified. Here we attempted to use a non-hypothesis driven, untargeted proteomic approach to quantify proteins in mosquito tissue. Work in other species has previously revealed ~20% of *An. gambiæ* genes and at least 8% *Ae. aegyæti* genes are rhythmically expressed over the 24-hour day [2].

---

**Fig 4.** *An. stephensi* proteogenomic analysis revealed evidence for the presence of *An. stephensi* peptide sequences that are not in the predicted *An. stephensi* (Indian strain) proteome but match predicted peptide sequences from species across a wide-range of taxa. Numbers listed as matches represent the number of ‘missing’ *An. stephensi* peptide groups found in the given taxonomic group. *Anopheles* species excludes the *An. stephensi* (SDA-500) strain. 

[https://doi.org/10.1371/journal.pone.0220225.g004](https://doi.org/10.1371/journal.pone.0220225.g004)
Here, in *An. stephensi*, we were able to detect rhythmic protein abundance levels in only 31 of the 1525 quantifiable proteins (2%). This is much lower than we expected from gene expression data from other mosquito species, but likely represents an underestimation of the true number of rhythmic proteins. One explanation for undetected rhythmic protein abundance is that low-concentration protein time courses were removed prior to analysis. These peptides may have represented protein abundance levels that were rhythmic, but dropped below the detection limits in our experimental runs at certain times-of-day. To our knowledge, this is the first untargeted quantitative proteomics performed in a mosquito species, and we reveal it can be used to reliably quantify a large number of proteins. We note, however, that some proteins may be rhythmic, or only appear at certain times-of-day, and this point should be considered when doing experimental design.

When we considered the homology of proteins here found to have rhythmic abundance levels, ~55% of identified *An. stephensi* rhythmic proteins have homologues in other species that have been determined, at the gene expression level, to be also rhythmic. This provides further evidence of the rhythmic nature of biological processes being conserved across species [35].

Finally, the present study revealed a number of potential errors in the current *An. stephensi* genome annotations and/or assembly. Untargeted proteomics could be leveraged to improve current genome annotations; however, proteomic reducibility, speed, and whole-proteome coverage are limited using our current technologies.

**Materials and methods**

**Biological material**

A lab colony of *An. stephensi* mosquitoes were maintained at ~60% relative humidity and 26°C on a 12 hr/12 hr LD cycle [11 hr full light, 11 hr darkness (0 lux) and 1 hr dawn and 1 hr dusk transitions]. Access to 8% (w/v) fructose was provided *ad libitum*. In three replicate time courses (with slightly different durations depending on available number of mosquitoes per batch, see Fig 1), mosquitoes were placed in individual containers (pots) and allowed to acclimate for several days. A pot of mosquitoes was euthanized on dry ice every four hours and placed in -80°C prior to tissue preparation. Heads were separated on dry ice from bodies (legs and wings were removed). Herein “body” describes the body of the mosquito with no head, wings, or legs.

**Sample preparation for the time series analysis**

A pool of 10 bodies was used per sample, solubilized in 250ul 8M urea 1% SDS and homogenized using a Precellys cell homogenizer (Bertin Instruments). The homogenization step comprises three steps of 40s at 5000rpm with a 10s pause; the overall procedure was repeated twice. A protein assay was performed using Pierce BCA protein assay kit, and a 50 μg protein equivalent was used for SDS-PAGE analysis. Samples were briefly run on SDS-PAGE gel for 10 min, extracted and digested using Shevchenko’s method [36]. Peptide extracts were then cleaned on SPE reverse phase Bond Elut LMS (Agilent). The samples were dried under low pressure (Speedvac from Thermo-Fisher) and stored at -20°C.

**HPLC-MS analysis**

The dried peptide samples were re-suspended in resuspension buffer (0.05%v/v trifluoroacetic acid in water) to a final concentration of 1 μg/μl. These samples were filtered using a Millex filter before subjecting to HPLC-MS analysis. Nano-HPLC-MS/MS analysis was performed using an on-line system consisting of a nano-pump (Dionex Ultimate 3000, Thermo-Fisher,
UK) coupled to a QExactive instrument (Thermo-Fisher, UK) with a pre-column of 300 μm x 5 mm (Acclaim Pepmap, 5 μm particle size) connected to a column of 75 μm x 50 cm (Acclaim Pepmap, 3 μm particle size). Samples were analyzed on a 90 min gradient in data dependent analysis (1 survey scan at 70k resolution followed by the top 10 MS/MS).

Proteomics, protein identification and quantification

Data from MS/MS spectra were searched using MASCOT Versions 2.4 (Matrix Science Ltd, UK) against *An. stephensi* (Indian strain Astel2.3) data stored in VectorBase [37, 38]. Search parameters included a maximum missed-cut value set to 2. The following features were used in all searches: i) variable methionine oxidation, ii) fixed cysteine carbamidomethylation, iii) precursor mass tolerance of 10 ppm, iv) MS/MS tolerance of 0.05 amu, v) significance threshold (p) below 0.05 (MudPIT scoring) and vi) final Mascot peptide score of 20. A complete dataset was analysed using MaxQuant v1.5.2.8 [39] assuming a Maxquant score of 45 and more.

For the time series quantification analysis Progenesis (version 4, Nonlinear Dynamics) was used for LC-MS label-free quantitation (S2 Table). Progenesis QI for proteomics software has been designed specifically to perform label-free quantitation and is capable of analyzing significant numbers of large data files due to its peak-modelling algorithm which reduces the data files by an order of magnitude without losing any information. This allows for the analysis of large data sets including large numbers of replicates that would otherwise be impractical to run. The software is enabled with a graphical user interface which allows MS data to be viewed in either two or three dimensions. This can help to verify if features have been quantified accurately. In brief, the basic software steps are as follows: (1) Alignment of runs to compensate for LC separation “between-run” variation, allowing like-for-like comparison of peptide signals; (2) Feature detection and quantitation using peak area method; (3) Peptide identification using the mascot search engine; and (4) Peptide/protein quantitation using the calculated abundance of the features to which identifications have been matched.

Only MS/MS peaks with a charge of 2+, 3+ or 4+ were considered for the total number of ‘Features’ (signal at one particular retention time and m/z) and only the five most intense spectra per ‘Feature’ were included. Normalization was first performed based on the median of the ion intensities of these sets of multi-charged ions (2+, 3+, and 4+). The associated unique peptide ion intensities for a specific protein were then summed to generate an abundance value, which was transformed using an ArcSinH function (a log transform is not ideal considering the significant amount of near zero measurements generated by the current method of detection). Based on the abundance values, within group means were calculated and from there the fold changes (in comparison to control) were evaluated. One-way ANOVA was used to calculate the p-value based on the transformed values. A larger dataset with samples from both heads and bodies were analyzed using Maxquant to generate a list of identified protein and peptides (S3 Table). False Discovery Rate (FDR) information is provided in Table 2.

Table 2. False Discovery Rate (FDR) information.

|                                      | *An. stephensi* | Decoy | FDR   |
|--------------------------------------|-----------------|-------|-------|
| Peptide matches above identity threshold | 11867           | 160   | 1.35% |
| Peptide matches above homology or identity threshold | 13300           | 248   | 1.86% |

https://doi.org/10.1371/journal.pone.0220225.t002
Homologue identification

To generate the gene names and orthologues in Table 1, the list of strain Indian (ASTEI) target proteins detected in the time course proteomics analysis translations was matched to strain SDA-500 (ASTE) homologues. Using this translation table, Indian strain proteins were matched to dipteran orthologous genes in OrthoDB [40, 41]. (ftp://cegg.unige.ch/OrthoDB8/Eukaryotes/Genes_to_Ogs/ODB8_EukOGs_genes_ALL_levels.txt.gz). Filtering was performed in a Jupyter notebook [42] using the Pandas (v0.18.1) Python library [43]. The notebook (https://figshare.com/s/dbb89cb869416979f60) is accessible on FigShare. Some gene names were manually supplemented using VectorBase [37, 38] or FlyBase [44].

Statistical analysis for rhythmic genes

In order to detect rhythmic protein abundance, we first only considered proteins where ANOVA revealed at least one time point is significantly different from the others (p < 0.1). Abundance data for those proteins was then processed with JTK_CYCLE [30] using the Meta-Cycle R package [31] to identify rhythmic proteins with a 24 hr period. Proteins were called rhythmic when their quantified protein abundance was determined by rhythmic by JTK_CYCLE (p < 0.1). We report at p = 0.09, q = 0.20 as our false discovery rate.

Proteogenomic analysis

A proteogenomic analysis was performed using the Mascot (Matrix science) package. Two sets of computed proteomes from VectorBase [37, 38] were utilized: (1) the An. stephensi proteome (Indian strain peptide sequences, Astel2.3 genaset with 11,789 entries); and (2) the entirety of arthropod proteomes stored in VectorBase (“All Vectors”, with >566,000 entries). This comprises 39 proteomes (S4 Table), both a second An. stephensi strain (SDA-500), other mosquito proteomes, snails, and other arthropod vectors such as sandflies, ticks, and kissing bugs.

We generated a subset of MS/MS features by removing MS/MS feature redundancy (keeping a maximum of the 5 most abundant peaks having the same masses and retention time). The merge .mgf file was generated using Progenesis. The dataset was searched against: (1) the An. stephensi proteome stored in VectorBase; and (2) against all the proteomes stored in VectorBase. We filter the identified peptide as follow: we only kept in both searches peptides having a Mascot score above identity and were ranked as first hit. We compared the two datasets and removed any specific peptide having the same score in both databases or showing similar peptide permutations such as isoleucine to leucine, which have indistinguishable mass spectrometry readings.

Supporting information

S1 Table. Identified peptides and proteins from untargeted proteomics.
(XLSX)

S2 Table. Progenesis output.
(XLSX)

S3 Table. Results of quantitative proteomics of An. stephensi bodies.
(XLSX)

S4 Table. List of reference species and gene build versions from VectorBase used for proteogenomic analyses.
(DOCX)
S5 Table. “Missing peptides” not found in An. stephensi, but with matches found in other species. (CSV)

S6 Table. BLAST analysis of An. stephensi genome assembly (Astel2-Indian strain) using the peptide sequences from proteomics analysis revealed missing genome annotations. Peptide transcript hit (100%), 100% identity to An. stephensi gene transcript with full query length using tblastn; Scaffold hit (100%), 100% identity to An. stephensi genome scaffold with full query length using tblastn; Wobbly scaffold hit (<100% hit) <100% identity to An. stephensi genome scaffold with 1 mismatch and with full query length ± 1 with tblastn; No hit, no hits (not transcript, scaffold, or wobbly scaffold hit) in An. stephensi genome using tblastn, missing in genome assembly. (CSV)

Acknowledgments

We thank Kimberly Prior, Aidan O'Donnell, and Ronnie Mooney for their assistance collecting and rearing mosquitoes during the time courses. We thank Sarah E. Reece for use of her lab space and equipment. We thank Michael Hughes, Gang Wu, and John Hogenesch for provision and assistance with JTK_CYCLE and MetaCycle. We thank Giles K.P. Bara for his useful comments on the manuscript.

Author Contributions

Conceptualization: Thierry Le Bihan, Samuel S. C. Rund.

Formal analysis: Thierry Le Bihan, Áine O'Toole, Paul V. Hickner, W. Augustine Dunn, Samuel S. C. Rund.

Funding acquisition: Samuel S. C. Rund.

Investigation: Lisa Imrie, Thierry Le Bihan, Benjamin Weise, Samuel S. C. Rund.

Methodology: Thierry Le Bihan.

Supervision: Samuel S. C. Rund.

Writing – original draft: Thierry Le Bihan, Áine O'Toole, Samuel S. C. Rund.

Writing – review & editing: Thierry Le Bihan, Paul V. Hickner, Samuel S. C. Rund.

References

1. Sinka ME, Bangs MJ, Manguin S, Chareonviriyaphap T, Patil AP, Temperley WH, et al. The dominant Anopheles vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic précis. Parasit Vectors. 2011; 4(1):89.

2. Rund SSC, Hou TY, Ward SM, Collins FH, Duffield GE. Genome-wide profiling of diel and circadian gene expression in the malaria vector Anopheles gambiae. Proc Natl Acad Sci USA. 2011; 108(32): E421—E30. https://doi.org/10.1073/pnas.1100584108 PMID: 21715657

3. Rund SS, Bonar NA, Champion MM, Ghazi JP, Houk CM, Leming MT, Syed Z, Duffield GE. Daily rhythms in antennal protein and olfactory sensitivity in the malaria mosquito Anopheles gambiae. Sci Rep. 2013; 3:2494. https://doi.org/10.1038/srep02494 PMID: 23986098

4. Balmert NJ, Rund SS, Ghazi JP, Zhou P, Duffield GE. Time-of-day specific changes in metabolic detoxification and insecticide resistance in the malaria mosquito Anopheles gambiae. J Insect Physiol. 2014; 64:30–9. https://doi.org/10.1016/j.jinsphys.2014.02.013 PMID: 24631664

5. Rowland M. Changes in the circadian flight activity of the mosquito Anopheles stephensi associated with insemination, blood-feeding, oviposition and nocturnal light intensity. Physiol Entomol. 1989; 14:77–84.
6. Mideo N, Reece SE, Smith AL, Metcalf CJE. The Cinderella syndrome: Why do malaria-infected cells burst at midnight? Trends Parasitol. 2013; 29(1):10–6. https://doi.org/10.1016/j.pt.2012.10.006 PMID: 23253515

7. Reece SE, Prior KF, Mideo N. The life and times of parasites: Rhythms in strategies for with-host survival and between-host transmission. J Biol Rhythms. 2017; 32(6):516–33. https://doi.org/10.1177/0748730417719894 PMID: 28845736

8. Rund SSC, O'Donnell AJ, Gentile JE, Reece SE. Daily rhythms in mosquitoes and their consequences for malaria transmission. Insects. 2016; 7(2):1–20.

9. Jiang X, Peery A, Hall AB, Sharma A, Chem X-G, Watershouse RM, et al. Genome analysis of a major urban malaria vector mosquito, Anopheles stephensi. Genome Biol. 2014; 15(9):459. https://doi.org/10.1186/s13059-014-0459-2 PMID: 25244985

10. Neafsey DE, Watershouse RM, Abai MR, Aganezov SS, Alekseyev MA, Allen JE, et al. Mosquito genomics. Highly evolvable malaria vectors: the genomes of 16 Anopheles mosquitoes. Science. 2015 Jan; 347(6217):1258522. https://doi.org/10.1126/science.1258522 PMID: 25554792

11. Valenzuela JG, Francischetti IM, Pham VM, Garfield MK, Ribeiro JM. Exploring the salivary gland transcriptome and proteome of the Anophlates stephensi mosquito. Insect Biochem Mol Biol. 2016; 72:717–32. PMID: 12826099

12. Fontaine A, Fusai T, Briolant S, Buffet S, Villard C, Baudelot E, et al. Anopheles salivary gland proteomes from major malaria vectors. BMC Genomics. 2012 Nov; 13:614. https://doi.org/10.1186/1471-2164-13-614 PMID: 23148599

13. Kurz S, Aoki K, Jin C, Karlsson NG, Tiemeier M, Wilson IB, et al. Targeted release and fractionation reveal glucuronylated and sulphated N- and O-glycans in larvae of dipteran insects. J Proteomics. 2015 Aug; 126:172–88. https://doi.org/10.1016/j.jprot.2015.05.030 PMID: 26047717

14. Pike A, Vadlamani A, Sandiford SL, Gacita A, Dimopoulos G. Characterization of the Rel2-regulated transcriptome and proteome of Anophletes stephensi identifies new anti-Plasmodium factors. Insect Biochem Mol Biol. 2016 Sep; 52:82–93. https://doi.org/10.1016/j.ibmb.2016.06.005 PMID: 24998399

15. Sikulu MT, Monkman J, Dave KA, Hastie ML, Dale PE, Kitching RL, et al. Proteomic changes occurring in the malaria mosquitoes Anopheles gambiae and Anopheles stephensi during aging. J Proteomics. 2015 Aug; 126:234–44. https://doi.org/10.1016/j.jprot.2015.06.008 PMID: 26100052

16. Prasad TS, Mohanty AK, Kumar M, Sreenivasanmurthy SK, Dey G, Nirujogi RS, et al. Integrating transcriptomic and proteomic data for accurate assembly and annotation of genomes. Genome Res. 2017 01; 27(1):133–44. https://doi.org/10.1101/gr.130868.115 PMID: 28003436

17. Champion MM, Sheppard A, Rund SSC, Freed SA, O'Tousa JE, Duffield GE. Qualitative and quantitative proteomics methods for the analysis of the Anophletes gambiae mosquito proteome. In Short Views on Insect Genomics and Proteomics 2016 (pp. 37–62). Springer, Cham.

18. Dinglasan RR, Devenport M, Florens L, Johnson JR, McHugh CA, Donnelly-Doman M, et al. The Anophletes gambiae adult midgut peritrophic matrix proteome. Insect Biochemistry and Molecular Biology. 2009; 39(2):125–34. https://doi.org/10.1016/j.ibmb.2008.10.010 PMID: 19038338

19. He N, Botelho JMC, McNall RJ, Belozerov V. Proteomic analysis of cast cuticles from Anophletes gambiae by tandem mass spectrometry. Insect Biochem Mol Biol. 2007; 37(2):135–46. https://doi.org/10.1016/j.ibmb.2006.10.011 PMID: 17244542

20. Kalume DE, Mobolaji O, Jun Z, Raghunath R, Shubha S, Nandan D, et al. A proteomic analysis of salivary glands of female Anophletes gambiae mosquito. Proteomics. 2005; 5(14):3765–77. https://doi.org/10.1002/pmic.200401210 PMID: 16127729

21. Khoury GA, Baliban RC, Floudas CA. Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. Sci Rep. 2011; 1:90.

22. Tanner S, Zhouxin S, Julio N, Liliana F, Guigo R, Briggs SP, et al. Improving gene annotation using peptide mass spectrometry. Proteome. 2007; 17:231–9.

23. Le Bihan T, Martin SF, Chirnside ES, van Ooijen G, Barrios-Llerena ME, O’Neill JS, et al. Shotgun proteomic analysis of the unicellular alga Ostreococcus tauri. J Proteomics. 2011; 74(10):2060–70. https://doi.org/10.1016/j.jprot.2011.05.028 PMID: 21635980

24. Millet C, Aushiannika D, Le Bihan T, Granneman S, Makovets S. Cell populations can use aneuploidy to survive telomerase insufficiency. Nat Commun. 2015; 6:8664. https://doi.org/10.1038/ncomms9664 PMID: 26489519

25. Guillaume E, Berger B, Aflolter M, Kussmann M. Label-free quantitative proteomics of two Bifidobacterium longum strains. J Proteomics; 2009; 72(5):771–84. https://doi.org/10.1016/j.jprot.2009.03.004 PMID: 19328873
26. Luber CA, Cox J, Lauterbach H, Fancke B, Selbach M, Tschopp J, et al. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. Immunity. 2010 Feb; 32(2):279–89. https://doi.org/10.1016/j.immuni.2010.01.013 PMID: 20171123

27. Jaffe JD, Berg HC, Church GM. Proteogenomic mapping as a complementary method to perform genome annotation. Proteomics. 2004; 4(1):59–77. https://doi.org/10.1002/pmic.200300511 PMID: 14730672

28. de Souza GA, Amtzen MO, Fortuin S, Schurch AC, Malen H, McEvoy CRE, et al. Proteogenomic analysis of polymorphisms and gene annotation divergences in prokaryotes using a clustered mass spectrometry-friendly database. Mol Cell Proteomics. 2011; 10(1):M110.002527—M110.

29. Ruggles KV, Tang Z, Wang X, Grover H, Askenazi M, Teubl J, et al. An analysis of the sensitivity of proteogenomic mapping of somatic mutations and novel splicing events in cancer. Mol Cell Proteomics. 2016 Mar; 15(3):1060–71. https://doi.org/10.1074/mcp.M115.06226 PMID: 26631509

30. Hughes ME, Hogenesch JB, Kornacker K. JTK_CYCLE: An efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. J Biol Rhythms. 2010; 25(5):372–80. https://doi.org/10.1177/0748730410379711 PMID: 20876817

31. Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB. MetaCycle: an integrated R package to evaluate periodicity in large scale data. Bioinformatics. 2016; 32(21):3351–3. https://doi.org/10.1093/bioinformatics/btw405 PMID: 27378304

32. Leming MT, Rund SSC, Behura SK, Duffield GE, O’Tousa JE. A database of circadian and diel rhythmic gene expression in the yellow fever mosquito Aedes aegypti. BMC genomics. 2014; 15(1):1128.

33. Ueda HR, Matsumoto A, Kawamura M, Iino M, Tanimura T, Hashimoto S. Genome-wide transcriptional orchestration of circadian rhythms in Drosophila. J Bio Chem. 2002; 277(16):14048–52.

34. Kumar A. An overview of nested genes in eukaryotic genomes. Eukaryotic Cell. 2009; 8(9):1321–9. https://doi.org/10.1128/EC.00143-09 PMID: 19542305

35. Filichkin SA, Breton G, Priest HD, Dharmawardhana P, Jaiswal P, Fox SE, et al. Global profiling of rice and poplar transcriptomes highlights key conserved circadian-controlled pathways and cis-regulatory modules. PLoS One. 2011; 6(6):e16907. https://doi.org/10.1371/journal.pone.0016907 PMID: 21694767

36. Shevchenko A, Henrik T, Jan H, V. OJ, Matthias M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 2007; 1(6):2856–60.

37. Giraldo-Calderón GI, Emrich SJ, MacCallum RM, Maslen G, Dialynas E, Topalis P, et al. VectorBase: An updated bioinformatics resource for invertebrate vectors and other organisms related with human diseases. Nucleic Acids Res. 2015; 43(Database issue):D707–13. https://doi.org/10.1093/nar/gku1117 PMID: 25510499

38. Lawson D, Peter A, Peter A, J. BN, V. BR, Ryan B, et al. VectorBase: A data resource for invertebrate vector genomics. Nucleic Acids Res. 2009; 37:583–7.

39. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 2008; 26(12):1367–72. https://doi.org/10.1038/nbt.1511 PMID: 19029910

40. Kriventseva EV, Rahman N, Espinosa O, Zdobnov EM. OrthoDB: the hierarchical catalog of eukaryotic orthologs. Nucleic Acids Res. 2007; 36(Database):D271—D5. https://doi.org/10.1093/nar/gkm845 PMID: 17947323

41. Waterhouse RM, Tegenfeldt F, Li J, Zdobnov EM, Kriventseva EV. OrthoDB: A hierarchical catalog of animal, fungal and bacterial orthologs. Nucleic Acids Res. 2013; 41(D1):358–65.

42. Kluyver T, Ragan-Kelley B, Pérez F, Granger B, Bussonnier M, Frederic J, et al. Jupyter Notebooks—A publishing format for reproducible computational workflows. 2016:87–90.

43. McKinney W. Data structures for statistical computing in python. 2010:51–6.

44. Attrill H, Falls K, Goodman JL, Millburn GH, Antonazzo G, Rey AJ, et al. Flybase: Establishing a gene group resource for Drosophila melanogaster. Nucleic Acids Res. 2016; 44(D1):D786—D92. https://doi.org/10.1093/nar/gkw1046 PMID: 26467479