Daily rhythms in antennal protein and olfactory sensitivity in the malaria mosquito *Anopheles gambiae*

Samuel S. C. Rund1, Nicolle A. Bonar1*, Matthew M. Champion2*, John P. Ghazi1, Cameron M. Houk1, Matthew T. Leming1, Zainulabeuddin Syed1 & Giles E. Duffield1

1Department of Biological Sciences and Eck Institute for Global Health, Galvin Life Science Center, University of Notre Dame, Notre Dame, IN 46556, 2Department of Chemistry and Biochemistry and Eck Institute for Global Health, Nieuwland Science Hall, University of Notre Dame, Notre Dame, IN 46556.

We recently characterized 24-hr daily rhythmic patterns of gene expression in *Anopheles gambiae* mosquitoes. These include numerous odorant binding proteins (OBPs), soluble odorant carrying proteins enriched in olfactory organs. Here we demonstrate that multiple rhythmically expressed genes including OBPs and takeout proteins, involved in regulating blood feeding behavior, have corresponding rhythmic protein levels as measured by quantitative proteomics. This includes *AgamOBP1*, previously shown as important to *An. gambiae* odorant sensing. Further, electrophysiological investigations demonstrate time-of-day specific differences in olfactory sensitivity of antennae to major host-derived odorants. The pre-dusk/dusk peaks in OBPs and takeout gene expression correspond with peak protein abundance at night, and in turn coincide with the time of increased olfactory sensitivity to odorants requiring OBPs and times of increased blood-feeding behavior. This suggests an important role for OBPs in modulating temporal changes in odorant sensitivity, enabling the olfactory system to coordinate with the circadian niche of *An. gambiae*.

*Anopheles gambiae* is the major African malaria vector. Insecticide and drug resistance highlights the need for novel malaria control strategies. *An. gambiae* exhibits daily rhythms in physiology and behavior that include flight, mating, sugar and blood-meal feeding and oviposition1–5. Olfaction is important for detection of blood-feeding hosts, sugar feeding sources and oviposition sites6. Our previously reported 48 hour analysis of circadian and diel (observed under light/dark [LD] cycle conditions) rhythmic gene expression found 25 known or putative olfactory genes in female heads. These rhythmic genes include 15 OBPs, 2 other chemosensory proteins and 2 takeout (TO) homologues7,8. Many of the OBPs that had rhythmic expression under LD conditions, had reduced amplitude rhythms in constant dark, or a complete loss of rhythmicity. This highlights the contribution of the LD cycle in shaping 24-hr rhythmicity in gene expression that has been proposed before in various taxonomic groups, and has been explicitly examined in *Drosophila*9.

OBPs, numbering about 51 in *Anopheles gambiae* Giles (Diptera: Culicidae) (www.vectorbase.org; release VB-2013-04), are encoded by some of the most abundantly expressed antennal transcripts10 and are critical for the transport of hydrophobic odorant molecules across the aqueous lymph space in the antenna to the dendrite surface10–14. In mosquitoes, OBPs bind to odorant molecules, and a subset have encoding gene expression enriched in female heads/olfactory organs relative to male tissues and/or female bodies and/or are down-regulated following blood-feeding. These findings provide further evidence of OBPs role in olfaction (only the female mosquito blood-feeds)10–16. *An. gambiae* and *Culex quinquefasciatus* OBP1 are functionally characterized and are proposed to bind to the host odorant indole. Moreover, the RNAi knockdown of OBP1 results in electrophysiological insensitivity to indole in *An. gambiae*1 and significant reduction in *Cx. quinquefasciatus*17. We have recently shown that gene expression rhythms in OBPs are driven in part by both the LD cycle and the endogenous circadian clock6, Sensory Appendage Protein (SAPP1, AGAP008051) and A10 (homologue to *Drosophila* antennal protein 10, AGAP008055) are associated with insect sensory organs such as the antennae, bear a hydrophobic pocket, and are proposed to have chemosensory function14. Similar to OBPs, and structurally similar to juvenile hormone binding protein, takeout proteins have a hydrophobic pocket, and are also thought to function as ligand carrier proteins involved in chemoreception19. *Drosophila* takeout plays a role in linking circadian output, temporal and food information to metabolic and feeding behavioral processes20. Knockdown of *takeout* RNA in vivo reduces *An. gambiae* blood-feeding1, thus demonstrating an important blood-feeding role of these proteins.
Tsetse flies, *Drosophila* and cockroaches have rhythmic daily changes in olfactory sensitivity, and *An. gambiae* exhibit diel and circadian rhythms of Blood-feeding behavior. Previous work demonstrated the presence of rhythmic proteins in mouse liver using 2D gel-electrophoresis and mass spectrometry for protein quantification and identification, respectively. Importantly, while it should not be assumed that a rhythm at the mRNA level will automatically be reflected at the protein abundance level, in many cases such coordination does occur. In our study of *An. gambiae* mosquitoes we hypothesized that rhythms in olfactory gene expression would correspond with rhythms in protein abundance, and that these rhythmic profiles would correspond with time-of-day changes in detection/sensitivity to host odors. We used quantitative multiple reaction monitoring (MRM) proteomics to measure changes in protein abundance in adult female olfactory tissues, comparing antennae and total head appendages (THA; maxillary palps, antennae and proboscis). Further, we measured olfactory responses induced by major host-derived chemicals from female adult antennae by electroantennograms (EAGs) to detect dose-dependent and time-of-day specific differences. Finally, we provide compelling behavioral evidence that confirms the rhythmic nature of flight and blood-feeding behavior in the laboratory, and that correlates strongly with our transcriptome, proteomic and physiological evidence for time-of-day specific changes in olfactory function.

**Results**

**Targeted quantitative proteomics.** We focused our proteomics study on rhythmic olfaction genes identified in our *An. gambiae* transcriptome study in olfactory tissues. We hypothesized that rhythmic levels of these transcripts will be translated into quantifiable protein rhythms, and lead to time-of-day specific changes in olfactory sensitivity and behavior. In order to assess the quality of our protein extraction, determine which olfactory proteins were likely 'visible' to a proteome, and generate empirical MS/MS spectra to be used for targeting, we performed data-dependent bottom-up proteomics on tryptic digests of mosquito heads, THAs and antennae (Fig. 1 and Fig. S1a). Single-dimensional nano-UHPLC MS/MS was chosen as it is identical to the level of separation analyzed by the targeted proteomics approach, thus allowing direct comparisons for verification and validation of targets. Our combined injections for bottom-up proteomics yielded a total of 1022 protein and 6157 peptide identifications at a 1% false discovery rate (Supplementary Dataset 1).

In order to determine if proteins identified by qualitative proteomics had rhythms specific to sensory tissues, we utilized targeted quantitative MRM proteomics to both identify and determine concentration in antennae and THAs. Biological material was collected every 4 hr for 24 hr from adult females maintained under strict LD cycle conditions (12 hr light: 12 hr dark including 1 hr dawn/dusk transitions). In antennae and THAs, we were able to defensibly quantify 21 of 25 targeted proteins, respectively, including 11 OBP and 5 other putative chemosensory proteins (Fig. 2, Fig. S3). The profiles of protein abundance for these proteins were found to be rhythmic. Detected OBP include OBP1 (AGAP003309), known to bind indole and DEET, and OBP20 (AGAP005208), known to bind DEET, 6-methyl-5-heptone-2-one and indole. Detected and rhythmic in our analysis, OBP1, 2 (AGAP003306), 3 (AGAP001409), 20, 22 (AGAP010409), 25 (AGAP012320), 26 (AGAP012321) and 47

![Figure 1](https://www.nature.com/scientificreports/images/202494/f1.png)

**Figure 1 | Targeted quantitative proteomics method.** Our experimental method is described. Briefly, adult female heads, antennae or THAs are pulverized, proteins extracted under denaturing conditions, clarified by centrifugation, digested with trypsin, quenched and desalted. Targeted quantitative proteomics was performed using multiple reaction monitoring (MRM) on a triple quadrupole (Q1rap 5500). MRM data were acquired in positive ion mode, using a Q1 of 100–1500 m/z and Q3 70–2000 m/z. Target ions were selected for peptide fragmentation and the data output was generated with Analyst software (Applied Biosystems). Median Fold Change vs. time, shown here after median transformation of the data. Note every protein had 2–3 underlying peptides used for quantification/qualification. An example of the three peptides used to quantify protein levels of OBP1 over 24 hr is shown. Targeted (quantitative MS/MS) proteomics is advantageous as it is highly specific, sensitive and linear with respect to quantity. In this work we demonstrate relative protein abundance rather than absolute quantities. Horizontal bars indicate day/night (white/black). See Fig. S1 for a detailed methodology and example results.
Figure 2 | Antennal protein rhythms correspond with RNA rhythms. (a) Quantitative proteomics reveals rhythms in antennal protein abundance that correspond highly with RNA expression profiles from whole-heads. Proteins are grouped into OBPs, non-OBP chemosensory proteins (AGAP007286, SAPPI1, A10, TO1, and TO2/3) and non-olfactory proteins (CYP6P3, RFeSP, PPO6, and VATI). All gene symbols as listed in VectorBase except for the following genes: RFeSP (homologue to Drosophila Rieske iron-sulfur protein, AGAP008955), A10 (homologue to Drosophila antennal protein 10, AGAP008055), VATI (predicted V-type proton ATPase catalytic subunit I, AGAP001587) and AGAP007286 (Ae. aegypti OBP43 homologue). Protein abundance normalized with tubulin. See Fig. S3 for protein rhythms in the second biological replicate antennae run and THAs. (b) Histogram of peak RNA expression phases in total heads and the corresponding peak antennal protein levels as determined by cosinor analysis from genes/proteins in panel A. (c) Cosinor analysis of antennae OBP protein levels from panel A (p < 0.001; acrophase ZT17.7); note OBP10 excluded as it has antiphasic expression compared to the other OBPs.

(AGAP007287) are reported to have gene expression enriched in female heads/olfactory organs relative to male tissues and/or female bodies^{10,12,13}. A recent RNA-seq experiment also revealed that many of these OBP genes are down-regulated in antennae following blood-feeding and as the mosquito transitions to oviposition behavior^{16}. These studies provide further evidence that these OBPs in particular are potentially involved in olfactory host-seeking behavior in females. Two other chemosensory proteins, SAPPI1 and A10, were also found to be rhythmic. Additionally, the takeout proteins TO1 (AGAP004263) and TO2/3 (AGAP012703/AGAP04268), which have been implicated in chemoreception and in blood-feeding behavior^{14,15}, were detected and rhythmic in antennae and THAs. Many protein rhythms were robust, with 3.2, 8.4, 4.5 and 5.3 peak-to-trough fold changes observed for OBP1, OBP26, TO1 and SAPPI1 antennae protein abundance, respectively.

In general, olfactory proteins were at their highest abundance at Zeitgeber time (ZT) 16 and lowest between ZT4 and ZT8 (ZT12, lights off; ZT0, end of dawn transition; Fig. 2 and Fig. S3). Protein profiles were compared with corresponding time-specific mRNA profiles, reanalyzed in 24 hr format from our 48 hr time course microarray analysis^{7}, and subjected to identical cosinor analysis to determine peak phase. Most of the proteins had a rhythmic phase lagging their corresponding RNA expression rhythm. With the exception of OBP10 (AGAP001189), which had antiphasic RNA and protein levels, OBP RNA rhythms peaked at ZT10.1 ± 0.9 (hr ± S.E.M) and protein rhythms at ZT16.8 ± 0.5 (measured by cosinor analysis), resulting in a peak OBP RNA to protein lag of 6.9 hr ± 0.7 hr (Fig. 2b,c). This common peak in OBPs was consistent across biological replicates and between antennae and THAs (cosinor analysis peak in antennae replicate 1, ZT18.4 ± 0.7 [group analysis, ZT17.7]; replicate 2, ZT16.9 ± 0.8 [ZT16.8]; and in THAs, 15.0 ± 0.9 [ZT14.9]; Fig. 2b–c and Fig. S3b–f). Such a large phase lag between RNA and protein rhythms has been observed previously^{26,32,33}, e.g. in mouse liver the RNA-protein lag of several clock components
and clock controlled genes, such as per1, per2, Id2 and Sdh1, is 6–9 hr\(^{24-26}\). The lag could be explained by the additional action of post-transcriptional\(^27\) and post-translational processes (e.g. protein phosphorylation and turnover), and our previously published gene expression analyses suggest that these mechanisms are in fact rhythmically regulated in An. gambiae\(^2\).

As part of our proteomics analysis we additionally targeted three proteins with rhythmic mRNA levels\(^3\) that are specifically involved in metabolic detoxification (CYP6P3, AGAP002865), immunity/cuticle pigmentation (PPO6, AGAP004977) and vesicular type H\(^+\) ATPase (VATPase) activity (VATI, AGAP001587): In all cases, protein levels were found to be rhythmic (Fig. 2, Fig. S3), and therefore suggests functional changes. As CYP6P3 metabolizes pyrethroids, such rhythmicity may confer time-of-day specific changes in insecticide resistance; PPO6 is a key enzyme involved in the melanization process, an important immune sequestering process; and changes in VATPase activity, amongst several things, contributes to synapse function and neurotransmitter recycling, see\(^7,8\). It is plausible that rhythmicity in this process might contribute additionally to the temporal changes observed in olfactory sensitivity (see electrophysiological analysis, below). In THAs, in addition to proteins reliably detected in antennae, we also defensively detected and profiled an additional three non-olfactory proteins which were rhythmic at the transcript level\(^7\) (Fig. S3d): The citric acid cycle enzyme isocitrate dehydrogenase (IDH; AGAP006660), the glutathione-S-transferase metabolic detoxification protein GSTD7 (AGAP004163), and Actin5C (AGAP000651). Note that the Drosophila Act5C gene promoter has been used frequently for transgenic mosquito experiments.

Not everything in our preparations had a protein profile with a peak during the early to mid-night phase (ZT12-20). In fact we observed profiles suggesting rhythmic peaks earlier in the 24 hr day; e.g. in THAs GSTD7 has a cosinor peak phase estimate of ZT9.4 and a peak expression measured at ZT4. There were several patterns of protein and RNA where this phase coordination was notably different: In both antennae and THAs RFeSP had a constitutively expressed RNA profile but rhythmic protein levels, while in THAs, GSTE3 (AGAP009197) displayed the opposite phenomenon, with rhythmic mRNA levels but corresponding constitutive protein levels. GSTE3 has been implicated in insecticidal detoxification\(^8\). Furthermore, in THAs the Actin5C protein profile was antiphasic to its encoding mRNA (cosinor peak phase estimate ZT3.0 and ZT16.7 for RNA and protein, respectively). Such a distinct absence of phase coordination between RNA and protein has been reported before\(^24,34\). For example, Reddy et al. similarly found half of cycling mouse liver proteins lacked a corresponding rhythmic mRNA; a phenomenon they attributed to post-transcriptional processes\(^24,35\). However, considering that our mRNA profiling was performed in total heads and protein analysis from specific olfactory tissues, we cannot exclude the possibility of tissue-specific mRNA rhythms that might correspond better with observed protein results.

Using proteomics we could not defensibly detect the obligatory co-receptor required for all odorant receptor (OR) function, Odorant Receptor Co-receptor Agam\Orco, also known as Or7 (AGAP0002560)\(^36-38\). RNA levels of Agam\Orco are rhythmic, peaking at ZT10 (prior to dusk and the onset of nocturnal behavioral activities involving olfaction)\(^2-5\). We previously hypothesized that rhythmic Agam\Orco protein levels could serve as a mechanism for rhythmic control of olfactory sensitivity\(^5\). However, immunoblot analysis revealed constitutive Agam\Orco levels (i.e. no significant differences in protein abundance were detected by Kruskal-Wallis ANOVA; cosinar analysis, n.s.; Fig. 3), indicating Agam\Orco is unlikely to gate rhythmic olfactory sensitivity. Note that we were unable to detect any odorant-specific ORs in our microarray analysis\(^39\) or proteomic analyses, presumably due to their low abundance\(^6\).

**Electrophysiological analysis.** We next determined if the temporal rhythms described in protein levels correspond with olfactory sensitivity. We used electroantennogram (EAG) analysis to measure the olfactory responses induced by host-derived odorant chemicals. At ZT4 (morning), ZT8 (afternoon), ZT12 (dusk) and ZT16 (night), mosquito antennae were challenged with major host-derived odorant chemicals (nonanal, indole, geranyl acetone, and a mixture of hexanoic acid, geranyl acetone, nonanal, indole, 3-methylindole and p-cresol). As several of the odorants are hydrophobic, it is expected that OBPs will be necessary for the mosquito to detect these stimuli. Since OBPs are rhythmic in protein abundance, we predicted rhythmic sensitivity in detection of these compounds. Indeed, we found time-of-day specific olfactory responses to all four stimuli, with sensitivity peaking at ZT16 (night) and least at ZT4 or ZT8 (day) (Fig. 4a–b). In all cases, the time-of-day specific differences in EAG amplitudes were comparable to the differences observed due to 10-fold changes in odorant concentration (mean peak-to-nadir difference in each time series: Geranyl acetone 44%, indole 50% and nonanal 61%; mean increase in response with each 10× increase in odorant concentration: Geranyl acetone 15%, indole 67%, and nonanal 81%). It is therefore plausible that the ≤8.4-fold rhythmic changes observed in OBP abundance (antennae mean ± SEM fold-change, 3.4 ± 0.4; THAs, 3.2 ± 0.6) could account for such differences in EAG responses. This is especially so given that in some cases multiple OBPs can bind a single odorant, and vice versa\(^11,14,15,17\). These findings are broadly consistent with our observed OBP protein rhythms (Fig. 2, Fig. S3) and mosquito behaviors described here (Fig. 6) and by others\(^5\). Specifically, protein expression and olfactory sensitivity were also lowest at ZT4-ZT8 and highest at ~ZT16, and blood-feeding and flight behaviors were higher at night than during the daytime. These data are particularly interesting for indole as there is significant evidence that OBP1 contributes to antennal sensing of this odorant\(^11,17\). OBP1 protein rhythm has a nadir at ZT4-ZT8 and a peak at ZT16 (Fig. 1, Fig. 2), which

![Figure 3 | Agam\Orco immunoblot analysis.](image-url)
corresponds with the lowest and highest sensitivity to indole in our electrophysiological measurements.

Next, we looked at olfactory responses to nonanal (host odorant that elicited highest EAG amplitude response from our panel) at ZT16 and at ZT8 from normally reared mosquitoes, but tested under identical lighting conditions. In order to control for any acute effects of light on influencing the odorant response, the ZT8 group was pretreated with 4 hr of darkness prior to EAG recording, and both ZT8 and ZT16 animals were tested under red light (Fig. 5a). It is plausible that light could influence olfactory function, either acting via the compound eye, or acting directly on the antennae via the blue light photoreceptor cryptochrome 1 (CRY1, AGAP001958), which is expressed in sensory organs of the mosquito39,40. Consistent with the experiments conducted under normal LD cycle conditions (Fig. 4a–b), this analysis revealed both a concentration dependent and a time-of-day effect: Mosquitoes at ZT16 retained a higher EAG sensitivity than dark-treated ZT8 mosquitoes. This indicates that reduced sensitivity at ZT8 is not an acute effect of light on the function of the antennae, but in fact a reflection of an underlying circadian clock and/or diel regulated mechanism (driven by the environmental 24 hr LD cycle)40. Further, 24 hr rhythmic changes in electrophysiological sensitivity persist in constant dark (DD) conditions in Drosophila and cockroaches41,42, and are driven by peripheral clocks located in the Drosophila antennae themselves43. Therefore, it is likely that the observed time-of-day changes in EAG sensitivity in An. gambiae are driven not by an acute effect of light or darkness, i.e. a masked response, but by an underlying clock and/or diel mechanism. This corresponds with our previous work that suggests that OBP gene expression is dependent upon both mechanisms working in concert44.

Finally, we tested the time-of-day specific olfactory sensitivity of mosquitoes to hexanoic acid, a hydrophilic host odor constituent that does not require OBPs for detection. Mosquito olfactory responses were measured with increasing doses of hexanoic acid, and compared to nonanal, which served as a positive control. As expected, responses to the nonanal control exhibited time-of-day sensitivity, peaking at ZT16, while responses to hexanoic acid did not change (two way ANOVA; Tukey post hoc tests, n.s.; Fig. 5b). Acid responses were however dose dependent (all concentrations are expected, responses to the nonanal control exhibited time-of-day sensitivity, peaking at ZT16, while responses to hexanoic acid did not change (two way ANOVA; Tukey post hoc tests, n.s.; Fig. 5b). Acid responses were however dose dependent (all concentrations are significantly different from each other; Tukey post hoc tests, p < 0.001). Earlier investigations of female Culex pipiens mosquitoes also did not reveal a time-of-day specific change in electrophysiological sensitivity to lactic acid45, which presumably does not require an OBP for detection.

Flight and blood-feeding behavior rhythms. Having established a clear time-of-the day modulation of abundant olfactory components in adult female An. gambiae using transcriptomic and proteomic approaches and sensory physiological evidence, we investigated if the An. gambiae strain from our laboratory maintained diel flight and blood-feeding behaviors typical of mosquitoes observed in the wild. Flight activity of host-seeking mosquitoes was monitored using an infra-red beam break system46, and our data revealed a clear onset of mosquito behavior at the beginning of the night (Fig. 6a), a finding consistent with laboratory and field observations47. Blood-feeding behavior has previously been reported to be under circadian
OBPs detected by microarray analysis\textsuperscript{7,8} (genomics) corresponds with peak protein abundance (proteomics), and is coincident with the time of increased olfactory sensitivity to host odors thought to require OBPs for detection (electrophysiology), and times of increased biting (blood-feeding) behavior. We also find that neither olfactory sensitivity to an odorant that does not require OBPs for detection (i.e. hexanoic acid), nor protein abundance of the olfactory co-receptor OR, Aga1Orco, changes across the 24 hr day. Rhythmic expression of OBPs peaks at the time of highest olfactory sensitivity to hydrophobic odorants (\textsim ZT16). Therefore, these results strongly suggest that rhythms in OBPs contribute to the changes observed in sensory and behavioral function. This is not surprising given that the knock-down of a single OBP, OBP1, results in a significant decrease in the sensitivity of major chemostimuli, including indole, in \textit{An. gambiae}\textsuperscript{11} and in \textit{Cx. quinquefasciatus}\textsuperscript{7}.

Though our data provides compelling evidence suggesting OBPs and other chemosensory proteins modulate olfactory sensitivity and behavioral outputs, we are aware that our quantitative transcriptome and proteome analyses did not detect another major chemosensory family, the ORs. Recent investigations have demonstrated a modulation of OR and OBP gene expression based on \textit{An. gambiae} physiology, and how such differences are coincident with a switch from host-seeking to oviposition behaviors. However, these changes in OR expression were modest as compared to the OBPs\textsuperscript{15}. Our own investigation into the role of ORs was inconclusive: The most abundant OR, Aga1Orco, did show a weak RNA rhythm in our microarray analysis\textsuperscript{7}, but we found no evidence of rhythmicity in protein abundance. Furthermore, additional downstream signal transduction components, as previously suggested\textsuperscript{9}, or rhythmic trafficking of olfactory components\textsuperscript{16} may also modulate olfactory responses.

The rhythmic blood-feeding responses described here are likely driven by several factors, including time-of-day changes in sensitivity to host odors (which may be driven by rhythmic OBP levels we describe here); rhythmic responses to CO\textsubscript{2}, as reported in the hematophagous \textit{Triatoma infestans}\textsuperscript{44}; and rhythmic protein abundance of behavioral/chemosensory factors, such as the takeout proteins TO1 and TO2/3, that we demonstrate to be rhythmic. Working alone or in concert with these above mentioned regulatory factors, it still remains a compelling hypothesis that rhythms observed in sensory organ OBP and takeout protein abundance could confer these dramatic time-of-day specific changes in odorant sensitivity and feeding behavior. The coincident times of peak protein abundance, olfactory sensitivity and behavior reflect the extraordinarily fine-tuned control of mosquito physiology, with OBP and other chemosensory protein abundance and high olfactory sensitivity up-regulated when needed (at night) and down-regulated when not required (daytime).

Our work highlights the important role of circadian/diel biology in the mosquito. Improved understanding of biological timing at the molecular level that underlies key physiological aspects of \textit{An. gambiae} may prove to be important for the successful implementation of existing or novel control methods and future experimental design. Further, greater understanding of the rhythmic nature of blood-feeding is important. There is growing evidence that the use of insecticide-impregnated bed nets is acting as a selective pressure, potentially modifying both age and genetic composition of the mosquito population\textsuperscript{45}. Thus, selection for mosquitoes that host-seek at times of the day when humans are not protected under bed nets may be occurring.

**Methods**

**Biological material.** \textit{An. gambiae} Pimpenera S form mosquitoes [MRA-861] were maintained at 85\% relative humidity and 27 ± 1°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). Time of day is reported in 24 hr Zeitgeber time (ZT) with ZT12 defined as time of lights off under the LD cycle, ZT0 defined as end of the dawn transition and ZT11 is defined as the start of the dusk transition. Access to 20\% high fructose corn syrup (HFCS) was provided ad libitum. For proteomics, three separate collections of ~30 mated but not blood-fed adult female 4–7 d old mosquitoes (i.e. host-seeking) were harvested on dry
Proteomics. Protein extraction and preparation. Mosquito antennae and THAs were processed by cryogenic freezing with liquid N₂ followed by manual pulverization with a 1.5 ml-tube pestle (USA Scientific, Ocala, FL) for 3 rounds of 15–30 s. Samples were then extracted with a mixture of equal volumes of 2.2,2, trifluoro-ethanol, 50 mM ammonium bicarbonate pH 8.0 (Fluka, Sigma-Adrich, St. Louis, MO) supplemented with 1 mM EDTA and 1 mM PMSF (Sigma, Sigma-Aldrich) 46. Extractions were performed for 15 min with shaking, and samples were clarified by centrifugation. Protein samples were precipitated by addition of 6-volumes of ice-cold acetone at −20°C for 1 hr, centrifuged, decanted and dried. Protein pellets were re-suspended, digested using 2,2,2, trifluoroethanol 46, re-suspended, reduced with 25 mM DTT (Sigma) for 1 hr at 56°C, then alkylated at room temp for 20 min with 35 mM iodoacetamide (Sigma). Samples were digested with addition of 500 ng of sequencing grade trypsin (Promega, Madison, WI) for 2 hr at 37°C with mild shaking, with an additional 1 µg added for overnight digestion; quenched by addition of trifluoroacetic acid (Optima, Thermo Fisher Scientific, Waltham, MA); lyophilized in a speed-vac concentrator; re-suspended in 0.1% TFA; and desalted with a C18 ZipTip (EMD Millipore, Billerica, MA) per manufacturer’s instructions.

Qualitative LC/MS/MS. The protein-digest extracts of An. gambiae antennae, THAs and heads were subjected to bottom-up LC/MS/MS analysis. Briefly, duplicate injections of antennae, THAs or head digests were separated on a 100 µm × 100 mm C18 BEH column (Waters, Milford, MA) over a 90 min gradient from 2–35% acetonitrile 0.1% formic acid (FA) (Burdick and Jackson, Honeywell, Morristown, NJ). MS and MS/MS data were acquired on a LTQ Velos Orbitrap instrument (Thermo Fisher Scientific) using a TOP8 method. Peak lists (mglf) were generated using RAW2MSM and subjected to database searches against the current version of the An. gambiae genome sequence from VectorBase (release VB-2013-04). The Paragon algorithm within Protein Pilot (ABSciex, Framingham, MA) was used for search and false-discovery rates were calculated with the PSPEP tool using the method of Elias et al 30. The results of the combined analysis are available in Supplementary Dataset 1, and represent one of the largest collections of sensory protein identifications in mosquito to-date. The combined injections for bottom-up proteomics yielded a total of 1022 protein and 6157 peptide identifications at a 1% false discovery rate (Supplementary Dataset 1) that compares in magnitude to the ~6,000 An. gambiae proteins comprehensively identified in Chaukady et al 31. No odorant receptors (ORs) were defensively identified at this stage after searching the bottom up generated development data. OR protein abundance was presumably below the detection limits of the experimental design; or the preparation used excluded their extraction. Note that OBP3 (AGAP001409) and OBP10 (AGAP001189) were only defensively detected in the antennae in the second biological replicate time course: For the purposes of presentation, these data are shown with the first replicate time course (Fig. 2). Raw protein identification data, including data from which the MRM’s were derived, have been deposited into the PeptideAtlas: http://www.peptideatlas.org/PASS/PASS00300.

MRM development, validation and acquisition. MRM transitions were determined largely from the empirical MS/MS data obtained from the bottom-up proteomics approach. Our analytical work was comparable to that employed in our previous MMR efforts 21–24 and in agreement with the guidelines described by the Aebersold group 35. A combination of empirical (MS/MS), MRM-initiated full-scan sequencing and published spectra were the source for the MRM peptide transitions. Specific transition development was performed using Skyline (MacCoss Lab) and MRMPilot (ABSciex) combined with manual refinement. An example of each type of peptide optimization is shown in Fig. S1b–g; including MRM-initiated full scan sequence confirmation performed as previously demonstrated 31.

Briefly, 4–9 transitions were chosen for each peptide used to describe a protein of interest for as many as 4 peptides per protein (several proteins only yielded one reproducibly detectable peptide for quantification). Transition validation and determination was made according to our previously published work and published sample validation methods 31. Transitions were compiled and reduced to quantifier and qualifier transition lists once optimized and validated by repeated injection, retention time (RT) prediction, RT cross-validation between disparate sample types (e.g. antennae, THAs and heads) and full-scan MS/MS confirmation from the bottom-up data and MRM-triggered MS/MS analysis. The final antennae list contained 214 transitions (Supplementary Dataset 2) with individual dwell times that were for
SCIENTIFIC REPORTS | 3 : 2494 | DOI: 10.1038/srep2494

the most part inversely proportional to the observed signal from the development data. This was performed to decrease the cycle-time thereby improving the sampling rate of the data (Supplementary Dataset 1). These were subsequently re-confirmed and tested using bulk mosquito-head extracts as a complete and complex sample stress-test. For each peptide analyzed, 3–4 transitions were chosen, similar to what we and others have observed35,39. Tubulins, due to their 24 hr constitutive RNA and rate of the data (Supplementary Dataset 1). These were subsequently re-confirmed the most part inversely proportionate to the observed signal from the development technical replicates (instrument variability) and less than 2-fold within biological reproducibility of biological replicates.

Peak Processing. Peak areas were integrated using MultiQuant (ABSciex) as described in Li et al. Briefly, 30 s RT windows were used, and a 3-point Gaussian smooth was applied to all transitions. Peak area integrations of the quantifier MRM transition were converted to area ratio/area response by dividing by the peak area of the associated internal standard peptides from β-tubulin. Additionally, identical numbers of antennae or THAs were processed at each time point ensuring reasonable application to all transitions. Peak area integrations of the quantifier MRM transition was used with pre-run solvent blanks injected. Mid-run washes and quality control peptide injections were performed every 12 injections. Including tuning, approximately 100 LC/MS/MS (MRM) acquisitions were acquired. An example chromatogram of a complete MRM trace, for THAs collected at ZT16, is shown in Fig. 51. Overall peak retention and reproducibility were excellent. Some runs exhibited a slight < 1 min (1–2%) systematic drift in retention-time (RT) throughout the >100 hr of acquisition, but this was readily corrected for with integration parameters. Most runs exhibited excellent stability. For example, RT stability for the β-tubulin peptide .FGQNLNADLR. (28) was 0.31%CV for all injections of antennae samples. This is typical of peptides observed in the antennae.

Behavioral assays. Individual mosquito locomotor/flight activity was measured with a Locomotor Activity Monitor 25 (LAM 25) system (TriKinetics, Waltham, MA), as previously detailed1. Briefly, individual mated but not blood-fed adult female 4–6 days post-emergence (i.e. host-seeking state) mosquitoes were placed in 25 × 150 mm clear glass tubes with access to 20% HFCS in the tubes provided ad libitum. Flight activity recorded as infrared beam breaks per minute. All recordings occurred in a light-proof box with its own lighting system in a 12 hr/12 hr LD cycle (1 hr full light, 11 hr darkness (0 Lux), and 1 hr dawn and 1 hr dusk transitions) with full light measured at the level of the LAM 25 between 69 and 119 lux. Mosquitoes were monitored for 7 full days. Locomotor flight activity was visualized in actogram format using ClockLab version 2.61 (Actimetrics, Wilmette, IL).

For blood-feeding preference assays, 4–8 days post-emergence mated adult female not blood-fed (i.e. host-seeking state) mosquitoes were separated into individual containers. Blood-feeding began the next morning. Mosquitoes were exposed (within inches) to human arm (the investigator’s) for 6 min. Mosquitoes were then anesthetized with CO2 and visually assayed for the presence of any blood in the abdomen to exclude potential biological circadian effects. Blood-feeding preference assays were performed with mosquitoes raised under reverse LD cycle conditions and then exposed to the human investigator at various phases of the circadian cycle. Similar time-of-day specific results were attained.

1. Das, S. & Dimopoulos, G. Molecular analysis of photic inhibition of blood-feeding in Anopheles gambiae. BMC Biol. 8, 23 (2008).
2. Gary Jr, E. R. & Foster, W. A. Diet timing and frequency of sugar feeding in the mosquito Anopheles gambiae, depending on sex, gonotrophic state and resource availability. Med. Vet. Entomol. 20, 308–316 (2006).
3. Barts, D. C., Torelli, M., Piaggio, G. & Cerami, A. Identification and expression of genes encoding odorant-binding proteins of the malaria-carrying mosquitoes Anopheles gambiae. BMC Genomics 13, 590–600 (2013).
4. Clements, A. N. The Biology of Mosquitoes. (CABI Publ, Oxon, 1999).
5. Takken, W. & Knols, B. G. Odor-mediated behavior of Afrotropical malaria mosquitoes. Annu. Rev. Entomol. 44, 131–157 (1999).
6. Barts, D. C., Torelli, M., Piaggio, G. & Cerami, A. Identification and expression of genes encoding odorant-binding proteins of the malaria-carrying mosquitoes Anopheles gambiae. Proc Natl Acad Sci USA 108, E421–E430 (2011).
7. Barts, D. C., Gentile, J. E. & Duffell, G. E. Extensive circadian and light regulation of the transcriptome in the malaria mosquito Anopheles gambiae. BMC Genomics 14, 218 (2013).
8. Wijnen, H., Naef, F., Boothroyd, C., Cläiränge-Chang, A. & Young, M. W. Control of daily transcript oscillations in Drosophila by light and the circadian clock. PLoS Biol. 4, e39 (2006).
9. Pitts, R. J., Rinkler, D. C., Jones, P. L., Rokas, A. & Zwiebel, L. J. Transcriptional profiling of chemosensory appendages in the malaria vector Anopheles gambiae reveals tissue- and sex-specific signatures of odor coding. BMC Genomics 12, 271 (2011).
10. Biessmann, H. et al. The Anopheles gambiae Odorant Binding Protein 1 (AgamOBP1) mediates indole recognition in the antennae of female mosquitoes. PLoS ONE 5, e9471 (2010).
11. Biessmann, H., Nguyen, Q. K., Le, D. & Walter, M. F. Microarray-based survey of a subset of putative olfactory genes in the mosquito Anopheles gambiae. Insect Mol. Biol. 14, 575–589 (2005).
12. Li, Z. X., Pickett, J. A., Field, L. M. & Zhou, J. I. Identification and expression of odorant-binding proteins of the malaria-carrying mosquitoes Anopheles gambiae and Anopheles arabiensis. Arch. Insect Biochem. Physiol. 58, 175–189 (2005).

11. Biessmann, H. et al. The Anopheles gambiae Odorant Binding Protein 1 (AgamOBP1) mediates indole recognition in the antennae of female mosquitoes. PLoS ONE 5, e9471 (2010).
41. Charlwood, J. D. et al. Lack of correlation between peripheral receptor sensitivity and the dynamic range proteome analysis of Drosophila melanogaster. *Nature* 400, 375–379 (1999).

42. Wanner, K. W. et al. Analysis of the insect os-1-like gene family. *J. Chem. Ecol.* 30, 889–911 (2004).

43. Yoshizawa, Y. et al. Ligand carrier protein genes expressed in larval chemosensory organs of Bombyx mori. *Insect Biochem. Mol. Biol.* 41, 545–562 (2011).

44. Barroso, R. B., Minoli, S. A. & Lazari, C. R. Circadian rhythm of behavioural responsiveness to carbon dioxide in the blood-sucking bug *Triatoma infestans* (Heteroptera : Reduviidae). *J. Insect Physiol.* 50, 249–254 (2004).

45. Moiroux, N. et al. Changes in Anopheles funestus biting behavior following universal coverage of long-lasting insecticidal nets in Benin. *J. of Infect Dis.* 206, 1622–1629 (2012).

46. Champion, M. M., Williams, E. A., Kennedy, G. M. & DiGiuseppe Champion, P. A. Detection of bacteriophage protein secretion using whole colony proteomics. *Molecular & Cellular Proteomics* 11, 596–604 (2012).

47. Elias, J. E. & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nature Methods* 4, 207–214 (2007).

48. Chaerkady, R. et al. A proteogenomic analysis of Anopheles gambiae using high-resolution Fourier transform mass spectrometry. *Genome Res.* 21, 1872–1881 (2011).

49. Li, Y., Wojcik, R., Dovichi, N. J. & Champion, M. M. Quantitative multiple reaction monitoring of peptide abundance introduced via a capillary zone electrophoresis–electrospray interface. *Anal. Chem.* 84, 6116–6121 (2012).

50. Chang, C. et al. Protein significance analysis in selected reaction monitoring (SRM) measurements. *Molecular & Cellular Proteomics* 11, M111.014662 (2012).

51. Anderson, L. & Hunter, C. L. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Molecular & Cellular Proteomics* 5, 573–588 (2006).

52. Fan, J., Preuss, F., Muskus, M. J., Bies, E. S. & Price, J. L. Drosophila and vertebrate casein kinases exhibit evolutionary conservation of circadian function. *Genetics* 181, 139–152 (2009).

53. Gilbert, M. R., Douris, N., Tongai, S. & Green, C. R. *Nocturnin* expression is induced by fasting in the white adipose tissue of restricted fed mice. *PLoS One* 6, e17051 (2011).

54. Pitts, R. J., Fox, A. N. & Zwiebel, J. L. A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *An. gambiae*. *Proc. Natl. Acad. Sci. USA* 101, 5058–5063 (2004).