Crepuscular Behavioral Variation and Profiling of Opsin Genes in *Anopheles gambiae* and *Anopheles stephensi* (Diptera: Culicidae)

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ABSTRACT We understand little about photopreference and the molecular mechanisms governing vision-dependent behavior in vector mosquitoes. Investigations of the influence of photopreference on adult mosquito behaviors such as endophagy and exophagy and endophily and exophily will enhance our ability to develop and deploy vector-targeted interventions and monitoring techniques. Our laboratory-based analyses have revealed that crepuscular period photopreference differs between *An. gambiae* and *An. stephensi*. We employed qRT-PCR to assess crepuscular transcriptional expression patterns of long wavelength-, short wavelength-, and ultraviolet wavelength-sensing opsins (i.e., rhodopsin-class G-protein coupled receptors) in *An. gambiae* and in *An. stephensi.* Transcript levels do not exhibit consistent differences between species across diurnal cycles, indicating that differences in transcript abundances within this gene set are not correlated with these behavioral differences. Using developmentally staged and gender-specific RNAseq data sets in *An. gambiae*, we show that long wavelength-sensing opsins are expressed in two different patterns (one set expressed during larval stages, and one set expressed during adult stages), while short wavelength- and ultraviolet wavelength-sensing opsins exhibit increased expression during adult stages. Genomic organization of *An. gambiae* opsins suggests paralogous gene expansion of long wavelength-sensing opsins in comparison with *An. stephensi*. We speculate that this difference in gene number may contribute to variation between these species in photopreference behavior (e.g., visual sensitivity).

KEY WORDS *Anopheles gambiae*, *Anopheles stephensi*, photopreference, crepuscular behavior, rhodopsin

Among deployable malaria control and prevention techniques, those targeting the primary host of *Plasmodium*—the vector mosquito—continue to constitute our most effective methods of intervention. The use of long-lasting insecticide-treated bed nets (Mittal et al. 2012) and indoor residual spraying (Kim et al. 2012), along with environmental management (Imbahale et al. 2012), have led to significant reductions in malaria-related morbidity and mortality in a number of disease-endemic countries (Fullman et al. 2013). However, we must be attentive to impacts on vector-targeted interventions of insecticide resistance (Weill et al. 2000, Reimer et al. 2008). In addition, the inexorable genesis of resistance and extended clearance times of malaria parasites following treatment with drugs, such as chloroquine, mefloquine, and most recently artemisinin, continue to compromise the utility of antimalarial drug-based interventions (Bray et al. 1998, Djimde et al. 2001, Dondorp et al. 2009, Alonso and Tanner 2013).

Creation of next-generation vector-targeted interventions that focus on aspects of the mosquito life cycle that are not targeted by present interventions (indoor residual spraying or IRS, and insecticide-treated bednets or ITNs) will depend, in part, on development of a broader understanding of the behaviors of vector mosquitoes. Many mosquito behaviors—including resting, foraging and feeding behaviors, olfactory responses, flight activity, and flight patterns—have been studied to identify prospective points of attack for next-generation vector-targeted interventions. Toward that end, we have begun to investigate illumination preferences of Anopheline mosquitoes.

Light traps are often used to monitor vector mosquito population compositions and densities (Overgaard et al. 2012, Tchouassi et al. 2012), and we anticipate that light sources could be incorporated into push-pull strategies (Takken 2010) for deflecting vector mosquitos from human dwellings. Still, light traps used to monitor biting rates have been known to provide conflicting results that can vary based on study methods, species observed, and geographical location (Mathenge et al. 2005, Mala et al. 2011). By understanding mosquito light preference in greater depth, we will expand our grasp of vector bionomics, and contribute to improvements in the use of light-based tools for

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monitoring vector populations and for the development of next-generation interventions that will contribute to decreasing the malaria burden in disease-endemic regions.

Anopheles funestus, *Anopheles stephensi*, and *Aedes aegypti* (L.) exhibit increased flight activity in dim-light settings compared with a setting of complete darkness, and the illumination intensities that stimulate flight vary among these species (Ribbands 1946, Manouchehri et al. 1976, Rowland 1989, Kawada et al. 2005). For instance, *An. stephensi* biting rates increase during nighttime hours, and house-entering behavior of *An. funestus* increases on moonlit nights (Ribbands 1946, Manouchehri et al. 1976, Rowland 1989). Mosquito house-entering and resting behaviors have been shown to be dependent on temperature microclimates, inside and outside of dwellings (Paaijmans and Thomas 2011). These resting preferences and illumination-influenced behaviors can impact malaria transmission by vector mosquitos and determine how accurately mosquito-monitoring techniques will reflect species prevalence. Integrative consideration of such bionomic factors has begun to influence the development of multiple interventions, including exposure to surface-applied malathion and fungal biocontrol agents, based on more extensive understanding of mosquito resting and flight behaviors (Perich et al. 2000, Mnyone et al. 2012).

While many innate behaviors have been well-characterized in many vector species, illumination preference is a mosquito behavior that has proven difficult to assay in lab and field settings. We have little molecular insight into possible mechanisms underlying illumination-dependent behavioral differences. For instance, multiple studies have reported conflicting results regarding the attractiveness to mosquitoes of blue and green wavelengths of light. Field studies of *Culex* spp. have reported attraction toward blue light, albeit the least intense of the visible wavelengths with regard to brightness in the study (Ali et al. 1989). Other field studies have concluded that a majority of mosquito species (among the genera *Anopheles*, *Aedes*, *Coquillettidia*, *Mansonia*, *Psorophora*, and *Uranotaenia*) prefers green wavelengths, although *Culex nigripalpus* females are reported to prefer blue wavelengths (Bentley et al. 2009).

On the other hand, laboratory-based experiments have shown that *Culex nigripalpus* feed for longer periods of time under illumination of 500 and 600 nm, within the green range of the visible spectrum (Burkett et al. 2012). Other species such as *Mansonia perturbans* are said to prefer wavelengths of 400–600 nm (blue–green range), while *An. stephensi* is said to be attracted to near-UV and incandescent light rather than to specific wavelengths (Wilton and Fay 1972, Browne and Bennett 1981). At present, we do not understand whether light preference differences among species, or potentially within species, depend on intrinsic genetic and molecular mechanisms, or on features of life history that engender habituation and learned preferences for specific wavelengths.

Within the order Diptera, molecular mechanisms underlying phototransduction and circadian rhythm have been investigated most extensively in *Drosophila melanogaster*, given the genetic and molecular tools available in this model organism (Montell 2012). We speculate that circadian variation in the expression of mosquito phototransduction genes may underlie diurnally variable mosquito behaviors. In the *Drosophila* head, over 150 genes associated with a variety of biological processes exhibit circadian oscillation in expression (Claridge-Chang et al. 2001). Hymenoptera, such as *Apis mellifera*, exhibit circadian fluctuations in expression of a green-sensitive *opsin* gene and an *arrestin* gene, each of which encodes phototransduction components, and their circadian rhythms may be controlled by a mechanism other than that mediated by Cryptochrome-2 (Sasagawa et al. 2003, Yuan et al. 2007).

Given the presence of 11 annotated *opsin* genes in the *An. gambiae* genome, *An. gambiae* has the largest number of *opsin* genes of any of the insects for which genome assemblies exist at present (Hill et al. 2002, Holt et al. 2002). This expanded *opsin* gene set has arisen, in part, due to an early duplication of long wavelength-sensitive *opsin* genes to create a set comprising six long wavelength-sensitive (λmax >500 nm) genes (*GPROP1*, *GROP3-7*)—in combination with one UV wavelength-sensitive (λmax <400 nm) *opsin* gene (*GPROP8*), one short wavelength-sensitive (λmax 400–500 nm) *opsin* gene (*GPROP9*), one functionally undefined *opsin* gene (*GPROP10*), and two *pteropsin* genes (*GPROP11*, *GRPOP12*; Spaethe and Briscoe 2004). To date, none of these *An. gambiae opsin* genes has been shown to exhibit statistically significant circadian variation in expression, although a number do vary in level over the 24-h circadian cycle (Rund et al. 2011). Behavioral analyses of *An. gambiae* have shown that manipulation of light can influence the timing of blood-feeding behavior (Das and Dimopoulos 2008). Finally, it has been proposed that variation between *An. gambiae* and *Ae. aegypti* in the localization of *opsin2* and *opsin8* expression within the compound eye may underlie species-specific behavioral patterns (e.g., photopreference in low light settings) that differ between these two vector mosquito species (Hu et al. 2009).

In this study we have developed a simple, laboratory-based assay to assess photopreference of *An. gambiae* and *An. stephensi*. We have employed these photopreference assays to determine that *An. gambiae* and *An. stephensi* exhibit different photopreferences, depending on the time of day and the illumination zone into which they are introduced. Subsequent qRT-PCR analysis fails to reveal significant diurnal differences in *opsin* gene expression, when comparing the two species. RNAseq analysis of *An. gambiae* opsins during four life stages indicates that one-half of the long wavelength-sensing opsins are expressed predominantly during larval stages and the other half during adult life-stages, while ultraviolet wavelength- and short wavelength-sensing opsins are expressed predominantly during adult stages. Further analysis of the organization of the long wavelength-sensitive *opsin* genes in the two species reveals that *An. gambiae* possess two more long wavelength-sensing opsins than *An. stephensi*, and we

speculate that this difference in gene number may contribute to the differences in photopreference that we observe in the two species.

Methods

Colony. *An. gambiae* G3 colony (courtesy of Dr. Flaminia Catteruccia, Harvard School of Public Health, Boston, MA) and *An. stephensi* Sind-Kasur strain Nijmegen (courtesy of Dr. Maria Mota, University of Lisbon, Lisbon, Portugal) were used for all experiments. All experiments were performed on mosquitoes 7–10 d post emergence, which were also aged 3–5 d post blood feeding and 1–3 d post egg laying. A photoperiod of 11:11 (L:D) h was maintained with 1 h dawn:dusk transitions between light and dark periods, with a constant temperature of 27°C and 80% relative humidity. Mosquitoes were fed 10% glucose solution ad libitum and were kept in the presence of the opposite sex throughout their life cycle.

Photopreference Assays. Photopreference assays were performed during the dawn:dusk and dusk:dawn transition periods. Assays were conducted using the arenas illustrated in Supp File 1 (online only). A 60" long, clear, plexiglass tube with a 2" interior diameter was used for the containment portion of the apparatus. For the trinary assays, photic zones were approximately 20" in length and were illuminated with 0 Lux, 100 Lux, or 400 Lux. Illumination levels were based on lux values of a lit room (Yu et al. 2007), and lux values obtained from observations outdoors during dawn and dusk hours in Chestnut Hill, MA. Binary assays consisted of a 30" dark zone (0 Lux) and a 30" illuminated zone (400 Lux). There was no temperature change within the tube throughout the course of the experiment, and the dark and illuminated zones of the tube remained at the same temperature. For each experimental run, approximately 50–75 mosquitoes were aspirated from the colony and introduced to the end of the tube employed for that run. A set of three biological replicates was completed for each pattern of introduction (i.e., illuminated end or dark end introduction). After mosquitoes were allowed to move throughout the tube for 20 min, mosquitoes were asphyxiated quickly by rapid exposure to high-concentration CO_2, to avoid alteration of resting patterns, and counts of male and female mosquitoes within each photic zone were then performed. The length of time used for each assay (20 min) was chosen as mosquito activity, i.e., the movement of mosquitoes among regions within the tube, did not change further beyond 20 min following the introduction of mosquitoes (data not shown).

Statistical Analysis. Statistical comparisons for the assessment of photopreference were performed using a Chi-Squared test to determine whether observed distributions deviated significantly from a random distribution. Statistical analyses were performed using Prism 5.0 software.

Collection of Samples and qRT-PCR of Selected Phototransduction Pathway Genes. All gene sequences, nomenclature, and identifiers are according to VectorBase VB-2013-12 (https://www.vectorbase.org; Last accessed 25 February 2015, Megy et al. 2012). qRT-PCR was performed for genes associated with known functions, including light detection and phototransduction pathways in both *An. gambiae* and *An. stephensi*. Samples were collected over a 48-h time period in order to encompass two complete diurnal L:D cycles. Collections were made every 4 h and consisted of approximately 10–15 female mosquitoes. Mosquito heads were immediately removed, and RNA was extracted using TriReagent (Sigma: St. Louis, MO), for use in subsequent analyses.

RPS7 (AGAP010592) gene expression was used as a reference for both species. Long wavelength-sensing (AGAP012982), short wavelength-sensing (AGAP010089), and ultraviolet wavelength-sensing (AGAP006126) genes were assayed for expression patterns, as compared to control genes, in both species. Sequences and concentrations of primers used for qRT-PCR can be found in Supp File 2 (online only). *An. stephensi* genes orthologous to those in *An. gambiae* were identified using local BLAST and manual annotation of the of the *An. stephensi* genome (VectorBase VB-2013-12). USB VeriQuest SYBR Green One-Step qRT-PCR Master Mix 2X (Affymetrix: Santa Clara, CA) was used to perform qRT-PCR. Cycling conditions were 50°C for 10 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 58°C for 30 s for *An. gambiae* (61°C for 30 s for *An. stephensi*). Reactions were run on a 7500 Fast Real-Time PCR System (Applied Biosystems: Grand Island, NY). qRT-PCR reaction products were subsequently sequenced to verify amplification of correct target sequences. All values were normalized to the highest expression value obtained for the given gene, for visualization purposes.

RNA Sequencing and Analysis. Male and female whole body RNAseq data sets from *An. gambiae* (GASUA strain) mosquitoes were obtained from Dr. Larry Zweibel and Dr. Jason Pitts (Vanderbilt University, Memphis, TN; Pitts et al. 2011). Those mosquitoes, which were reared with a photoperiod of 12:12 (L:D) h in 75% humidity, were collected for sequencing at Zeitgeber time 10–12, and were therefore exposed to illumination preceding collection of RNA. We collected two biological replicates at the same time points as Pitts et al. (2011), i.e., first (L1) and third (L3) instar larvae, as well as single biological replicates of adult males and females (whole body) of *An. gambiae* G3 to compliment the Vanderbilt University data set. We collected only single adult replicates as our goal was to validate expression levels reported by Pitts et al. (2011), rather than define statistically significant differences in transcriptional expression among life stages. RNAseq data sets have been deposited in the European Nucleotide Archive under the SRA accession PRJEB5712. RNA extraction and sequencing of these collections were performed by Otogenetics Corp. (Norcross, GA) and the Broad Institute (Cambridge, MA). All RNA-seq data were aligned to *An. gambiae* P3 assembly, from VectorBase VB-2013-12, using Tophat2 (Kim et al. 2013). FPKM values and comparisons between samples were performed using

Cufflinks-Cuffdiff2, and the subsequent heatmap was visualized using CumberBund (Trapnell et al. 2013). Genes analyzed included all long wavelength-sensing opsins *GPROP1* (AGAP013149), *GPROP3* (AGAP012982), *GPROP4* (AGAP012985), *GPROP5* (AGAP001162), *GPROP6* (AGAP001161), *GPROP7* (AGAP002462), ultraviolet wavelength-sensing opsin *GPROP8* (AGAP006126), short wavelength-sensing opsin *GPROP9* (AGAP010089), an unknown wavelength-sensing opsin *GPROP10* (AGAP007548), and the two pteropsins *GPROP11* (AGAP002443) and *GPROP12* (AGAP002444).

Results and Discussion

Determination of Photopreferences in *An. gambiae* and *An. stephensi*. First, we measured photopreference characteristics of *An. gambiae* and *An. stephensi* to determine whether there are distinctions between the two species. We developed an assay that assesses the photopreference of *An. gambiae* using a binary choice arena (0 Lux vs. 400 Lux, Fig. 1, Table 1, Supp File 1 [online only]). Introduction of mosquitos into the illuminated end of the apparatus during either dawn or dusk crepuscular periods reveals that females exhibit a significant preference for darkness, while males exhibit no reference between illumination and darkness (Fig. 1A andE). Binary choice assays in which *An. gambiae* was introduced into the darkened end of the apparatus reveal that males and females exhibit a significant preference for resting in darkness (Fig. 1C andG).

Analogous experiments with *An. stephensi* reveal that females prefer the illuminated portion of the apparatus when added to the illuminated end of the apparatus at dawn, while males prefer darkness (Fig. 1B). When introduced into the illuminated end of the apparatus, females exhibit a preference for illumination at dusk, while males no longer display any illumination preference (Fig. 1F). When added to the darkened portion of the apparatus at dawn, *An. stephensi* females lack any discernible photopreference, while males display a preference for darkness (Fig. 1D). When introduced into darkened end of the apparatus at dusk, *An. stephensi* males exhibit no preference, while females exhibit a preference for the illuminated portion of the apparatus (Fig. 1H).

The differences we observed between *An. gambiae* and *An. stephensi* photopreferences are consistent with differences observed in past studies of each species in other physical settings (Jones et al. 1967, Rowland 1989). Female *An. gambiae* generally exhibit a significant preference for a darkened photic zone, which can be attributed to an active avoidance of increased illumination. The active avoidance of illumination by *An. gambiae* females, when they are introduced to the 400 Lux end of the arena (Fig. 1A and E), indicates an avoidance of the light rather than a simple, consistent preference toward the end of the apparatus into which the mosquitos are introduced. Given that previous studies of *An. gambiae* indicate that peak flight activity occurs at the dawn and dusk hours, the possibility that *An. gambiae* are not actively moving within our apparatus is unlikely (Jones et al. 1967).

Interestingly, *An. stephensi* photopreference differs greatly from that of *An. gambiae.* Female *An. stephensi* prefer the 400 Lux region of the apparatus in all conditions, except when introduced into 0 Lux at dawn, when no significant preference was observed. This suggests a requirement for increased illumination to perform visual-based behaviors, such as identifying a feeding source, an oviposition site, or a mating swarm, or for achieving increased visual acuity. Male *An. stephensi* exhibit a preference for darkness or no preference, for all patterns of introduction, similar to findings for *An. gambiae* males. This suggests that light preference may be less important for *Anopheline* males in the processes of finding mates and food sources. In order to further validate the distinctions in photopreferences we observe between the two species in a binary choice assay, we subsequently conducted trinary choice assays.

Assessment of *An. gambiae* photopreference in a trinary choice assay (0 Lux vs. 100 Lux vs. 400 Lux, Fig. 2, Table 2), which allows for greater delineation of photopreference, illustrates that females and males prefer 100 Lux illumination during dawn and dusk crepuscular periods, when introduced to the 400 Lux end of the apparatus (Fig. 2A andE). When the assay was repeated with the introduction of mosquitos into the 0 Lux end of the apparatus, both sexes of *An. gambiae* prefer to remain in the darkened end of the apparatus during both crepuscular periods (Fig. 2C andG). *An. stephensi* display tendencies to rest in 400 and 100 Lux regions of the apparatus, instead of the nonilluminated region, when introduced to the 400 Lux-illuminated region of the apparatus during dawn or dusk (Fig. 2B andF). Following introduction into the darkened end of the apparatus during dawn, *An. stephensi* males and females remain in the darkened region (Fig. 2D). Females exhibit no preference following introduction into the darkened end during dusk, and males exhibit significant preference toward the 100 Lux-illuminated region when introduced in the same manner (Fig. 2H).

With the availability of a photic zone with intermediate illumination in which to rest, both *An. gambiae* and *An. stephensi* photopreferences are altered compared with those measured in the binary photo assay format. Female and male *An. gambiae* exhibit strong preferences for darkness when introduced to the 0 Lux end of the apparatus, as in the binary photo assay. However, both sexes prefer to rest in the intermediate (100 Lux) illumination zone when introduced to the 400 Lux zone (Fig. 2A andE). These results indicate *An. gambiae* males and females still actively avoid the most intensely illuminated region of the apparatus, but do not necessarily prefer complete darkness. Rather, the avoidance of 400 Lux illumination, as seen in the binary assays, can be achieved by resting in the 100 Lux region rather than the 0 Lux region of the arena. The differing *An. stephensi* trinary preference data indicate a strong preference for an illuminated area when introduced to the 400 Lux end of the arena (Fig. 2B andF),

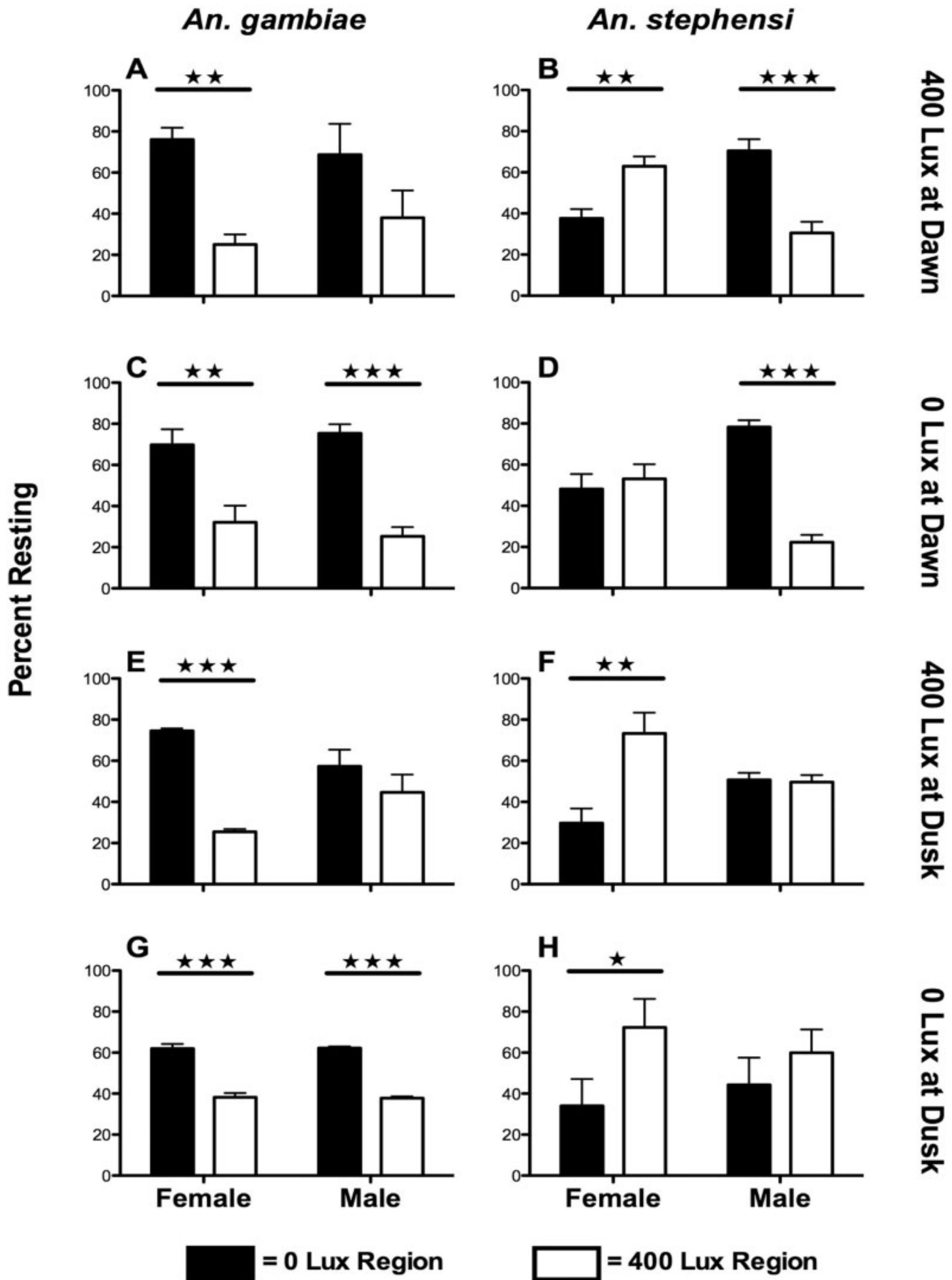

Fig. 1. *An. gambiae* and *An. stephensi* binary photopreference. Bar graphs depict percent of mosquitos resting in specific photic regions (±SEM, $N = 3$) for each experiment. Left and right columns depict *An. gambiae* and *An. stephensi* resting patterns for each condition, respectively, with males and females being depicted within each column. Dawn and dusk refer to relative crepuscular period. Right hand titles indicate introduction site followed by relative crepuscular period. Black bars represent mosquitos resting in the 0 Lux region of the tube at the end of the experiment, and open bars represent those resting in the 400 Lux region. (A and B) Introduction into 400 Lux region at dawn. (C and D) Introduction into 0 Lux region at dawn. (E and F) Introduction into 400 Lux region at dusk. (G and H) Introduction into 0 Lux region at dusk. ★$P < 0.05$, ★★$P < 0.01$, ★★★$P < 0.001$. Tabulations can be found in Table 1.

Table 1. *An. gambiae* and *An. stephensi* binary photopreference data

Zeit. time	Int Site	*An. gambiae*				*An. stephensi*			
		Female	Male	Female	Male	Female	Male	Female	Male
Dawn	400	76.1 ± 3.3	68.7 ± 8.7	25.1 ± 2.9	38.0 ± 7.7	37.6 ± 2.6	70.5 ± 3.3	63.0 ± 2.8	30.5 ± 3.2
Dawn	0	69.8 ± 4.3	75.4 ± 2.6	32.09 ± 4.71	25.3 ± 2.6	48.2 ± 4.2	78.3 ± 2.0	53.2 ± 4.1	22.3 ± 2.1
Dusk	400	74.6 ± 0.7	57.3 ± 5.0	25.5 ± 0.7	44.6 ± 5.0	29.7 ± 4.1	50.7 ± 2.0	73.3 ± 5.8	49.6 ± 2.0
Dusk	0	62.0 ± 1.3	62.2 ± 0.5	38.2 ± 1.2	37.8 ± 0.5	34.1 ± 7.5	44.3 ± 7.6	72.3 ± 8.0	59.9 ± 6.6
		0 Lux		400 Lux		0 Lux		100 Lux	
		Photic preference zone							

Tabulation of results presented in Figure 1. Zeitgeber time and Introduction site are presented in the left-hand columns, with photic regions represented with 0 Lux and 400 Lux. Values are percent resting in respective region ± SEM.

consistent with the hypothesis that *An. stephensi* mosquitos require more intense light in order to experience visual perception comparable with that of *An. gambiae*. These data are also consistent with past findings that *An. stephensi* exhibits increased flight activity in a dim-light setting compared with complete darkness (Rowland 1989).

The photopreference differences that we define in binary and trinary assays indicate that our simple photopreference arena—the first of its kind for vector mosquitos—is adequate for assessing differences in photopreferences between species, in a laboratory setting. The simple fabrication, low monetary cost, and ease of transportation and setup of the assay arena imply that the assay could be performed with field-captured mosquitoes in a field setting. This strategy would reduce the need to create stable laboratory colonies of field-caught mosquitoes for photopreference behavioral assays and may enable more accurate analysis of a given species' photopreference in the field. Photopreference is of interest as it may inform how insecticides are applied in the field, in addition to expanding our understanding of vector photobiology. Better knowledge of mosquito photopreference may enable the application of insecticides to more specific areas of interest in the home and in the field, in conjunction with control efforts, rather than the use of broad-pattern application that covers many areas without biological relevance to the vector-targeted control. Current insecticide application methods, such as indoor residual spraying, often involve treating the entirety of a dwelling and leaving a residual coating of insecticide for months after treatment. A given vector mosquito population might experience minimal contact with many of these treated surfaces, depending on its resting patterns within dwellings. By understanding these resting patterns in greater depth, the amount of insecticide needed for spraying may be reduced and better allocated to increase vector contact with insecticides and thereby increase the effectiveness of residual insecticide treatment methods.

Diurnal Variation of *Opsin* Gene Expression. Previous studies have shown that larval swimming behavior in the ascidian *Ciona intestinalis* can be altered by knocking down *Ci-Opsin1*, which results in reduced photoresponsiveness (Inada et al. 2003). Given these findings, we chose to determine whether diurnal transcriptional expression patterns of selected *opsin* gene superfamily members in *An. gambiae* and *An. stephensi* are correlated with distinct diurnal photopreferences we observe in these species. The *An. gambiae* haploid genome contains 11 annotated *opsin* genes (Hill et al. 2002, Holt et al. 2002). Eight of the 11 genes have attributable functions, and are defined as long wavelength-sensing, short wavelength-sensing, and ultraviolet wavelength-sensing *opsin* genes. Our Reciprocal Best Blast analysis and manual annotation of the *An. stephensi* genome (VectorBase VB-2013-12) using *An. gambiae opsin* genes as query sequences led to the identification of four long wavelength-sensing *opsin* genes, one short wavelength-sensing *opsin* gene, and a single ultraviolet wavelength-sensing *opsin* gene within the *An. stephensi* genome. The organization of a subset of *An. gambiae opsin* genes and homologous genes in *An. stephensi* is depicted in Figure 3. On chromosome 2R, *An. gambiae* possesses four long wavelength-sensing *opsin* genes within a gene cluster (*GPROP3, GPROP4, GPROP5, GPROP6*; Fig. 3). *An. stephensi* contains a similar cluster that includes only three long wavelength-sensing genes. The difference between these clusters in the two genomes is an apparent *opsin* gene duplication and inversion of *GPROP4* in *An. gambiae*. In other organisms, mainly primates, increased range of wavelength sensing and trichomatic color vision have been correlated with evolutionary duplications of long wavelength-sensing and medium wavelength-sensing *opsin* genes (Dulai et al. 1999). Therefore, the increased number of long wavelength-sensing *opsin* genes in *An. gambiae* as compared with *An. stephensi* may contribute mechanistically to differences in their photopreference behaviors.

We assessed only the long wavelength-sensing *GPROP3* for diurnal expression variation for a number of reasons. First, previous studies by Rund et al. (2011) did not suggest diurnal variation in the expression of any opsin (Rund et al. 2011). Second, due to sequence conservation among the long wavelength-sensing *opsin* gene set we have defined, *GPROP3* was the only long wavelength-sensing *opsin* gene that could be verified specifically as being expressed using qRT-PCR in *An. gambiae*.

The *GPROP3*, *GPROP8*, and *GPROP9* genes in *An. gambiae*, which are predicted to detect long wavelengths, ultraviolet wavelengths, and short wavelengths, respectively, exhibit no significant diurnal variation in

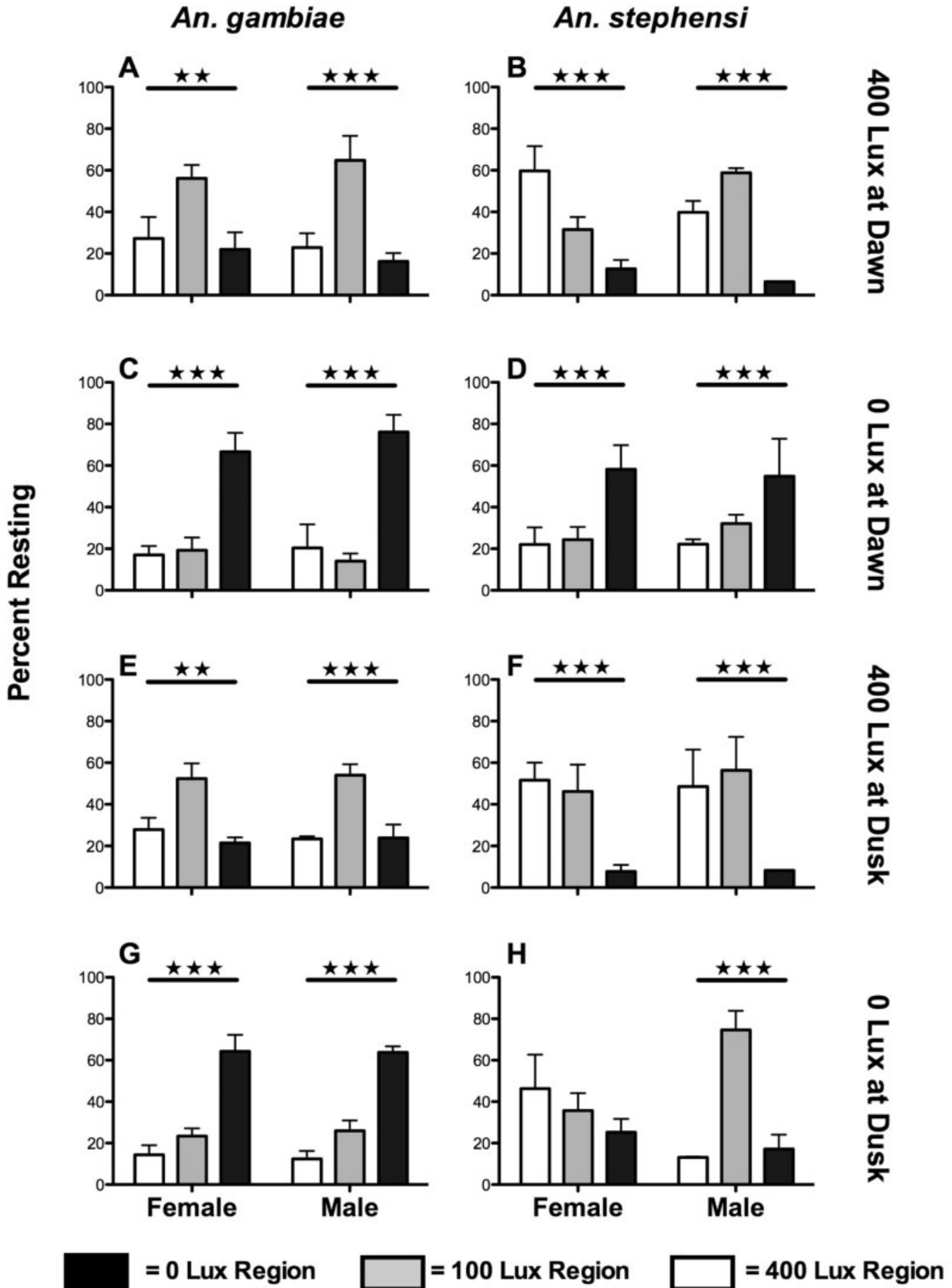

Fig. 2. *An. gambiae* and *An. stephensi* trinary photopreference. Bar graphs depict percent of mosquitos resting in specific photic regions (±SEM, $N=3$) for each experiment. Left and right columns depict *An. gambiae* and *An. stephensi* resting patterns for each condition, respectively. Dawn and dusk refer to relative crepuscular period. Right hand titles indicate introduction site, followed by relative crepuscular period. Black bars represent mosquitos resting in the 0 Lux region of the tube at the end of the experiment, gray bars represent those resting in the 100 Lux region and open bars represent those resting in the 400 Lux region. (A and B) Introduction into 400 Lux region at dawn. (C and D) Introduction into 0 Lux at dawn. (E and F) Introduction into 400 Lux at dusk. (G and H) Introduction into 0 Lux at dusk. ★$P<0.05$, ★★$P<0.01$, ★★★$P<0.001$. Tabulations can be found in Table 2.

Table 2. *An. gambiae* and *An. stephensi* trinary photo preference data

Zeitgeber time	Introduction site	*An. gambiae*			*An. stephensi*		
Females							
Dawn	400	27 ± 6.0	56.1 ± 3.7	22.0 ± 4.7	59.7 ± 6.9	31.5 ± 3.5	12.6 ± 2.5
Dawn	0	17.1 ± 2.5	19.3 ± 3.5	66.7 ± 5.2	22.0 ± 4.8	24.4 ± 3.5	58.2 ± 6.7
Dusk	400	27.9 ± 3.6	52.4 ± 4.2	21.5 ± 1.5	51.6 ± 4.9	46.2 ± 9.5	7.7 ± 1.8
Dusk	0	14.4 ± 2.6	23.4 ± 2.2	64.3 ± 4.6	46.3 ± 9.5	35.7 ± 4.9	25.2 ± 3.7
Males							
Dawn	400	22.9 ± 4.0	64.8 ± 6.8	16.2 ± 2.3	39.8 ± 3.2	58.8 ± 1.3	6.5 ± 0.0
Dawn	0	20.4 ± 6.6	14.1 ± 2.1	76.1 ± 4.8	22.2 ± 1.3	32.1 ± 2.4	54.9 ± 10.4
Dusk	400	23.4 ± 0.7	54.1 ± 3.0	23.9 ± 3.7	48.6 ± 10.2	56.4 ± 9.3	8.9 ± 0.1
Dusk	0	12.3 ± 2.9	25.9 ± 2.9	63.8 ± 1.7	13.1 ± 0.2	74.6 ± 5.3	17.2 ± 4.0
		400 Lux	100 Lux	0 Lux	400 Lux	100 Lux	0 Lux
		Photic preference zone					

Tabulation of results presented in Figure 3. Zeitgeber time and introduction site are presented in the left-hand columns, with photic regions represented with 0, 100, and 400 Lux. Values are percent resting in respective region ±SEM.

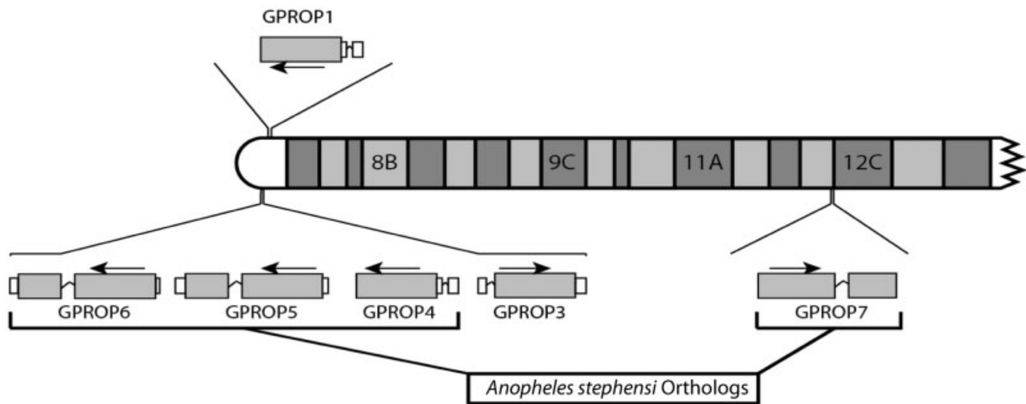

Fig. 3. Long wavelength opsin gene organization on *An. gambiae* chromosome ARM 2R. Five of the six long wavelength-sensing *opsin* genes cluster toward the telomeric end of chromosome 2R in *An. gambiae*. This gene number contrasts with the four orthologous long wavelength-sensing *opsin* genes present in *An. stephensi*.

transcription during the 48-h time period assayed (Fig. 4 A,C andE). Among the orthologous genes in *An. stephensi*—annotated as *LW*, *UV*, and *SW* for putative long wavelength-, ultraviolet wavelength-, and short wavelength-responsive *opsin* genes, respectively—the *LW* and *SW* genes fail to exhibit striking diurnal variation in transcription (Fig. 4B andF). The *UV* gene transcript levels increase during the dusk crepuscular period compared to levels during other intervals of Zeitgeber time (Fig. 4D). As there are no significant differences in diurnal expression patterns for *opsin* genes we assayed, we can reject the hypothesis that variation in expression of the *opsin* genes assayed is correlated with variations in photopreference that we observe between these two species. Although the transcript levels do not vary throughout diurnal phases, it is possible that protein levels may vary due to translational or post-translational regulation. However, assessment of those possibilities lies beyond the scope of our analysis. Alternatively, as subcellular localization of some opsins in the photoreceptor cells of *Ae. aegypti* and *An. gambiae* has been described, changes in this subcellular localization, again beyond the scope of our analysis, may account for variability in photopreference between species (Hu et al. 2009, 2011, 2013).

Developmental Expression and Evolution of *Opsins* in *An. gambiae*. The difference we observe in long wavelength-sensing *opsin* gene number in *An. gambiae* and *An. stephensi* led us to question the potential functional significance the existence of six long wavelength-sensing opsin genes in *An. gambiae* and only four long wavelength-sensing opsin genes in *An. stephensi*. To investigate this question in *An. gambiae*, we utilized RNAseq analysis to assess expression of each of the 11 *opsin* superfamily gene members during first and third larval instars, and in female and male adults (Fig. 5, Supp File 3 [online only]). Three annotated long wavelength-sensing *opsin* genes—*GPROP1*, *GPROP3*, and *GPROP4*—are expressed more highly during adult stages, and long wavelength-sensing *opsin* genes *GPROP5*–*GPROP7* all exhibit increased expression during larval stages, consistent with previous findings from microarray-based expression analyses (Marinotti et al. 2006, Rund et al. 2011). *GPROP11* and *GPROP12*, pteropsins, are also expressed at low levels during all life stages studied. In contrast,

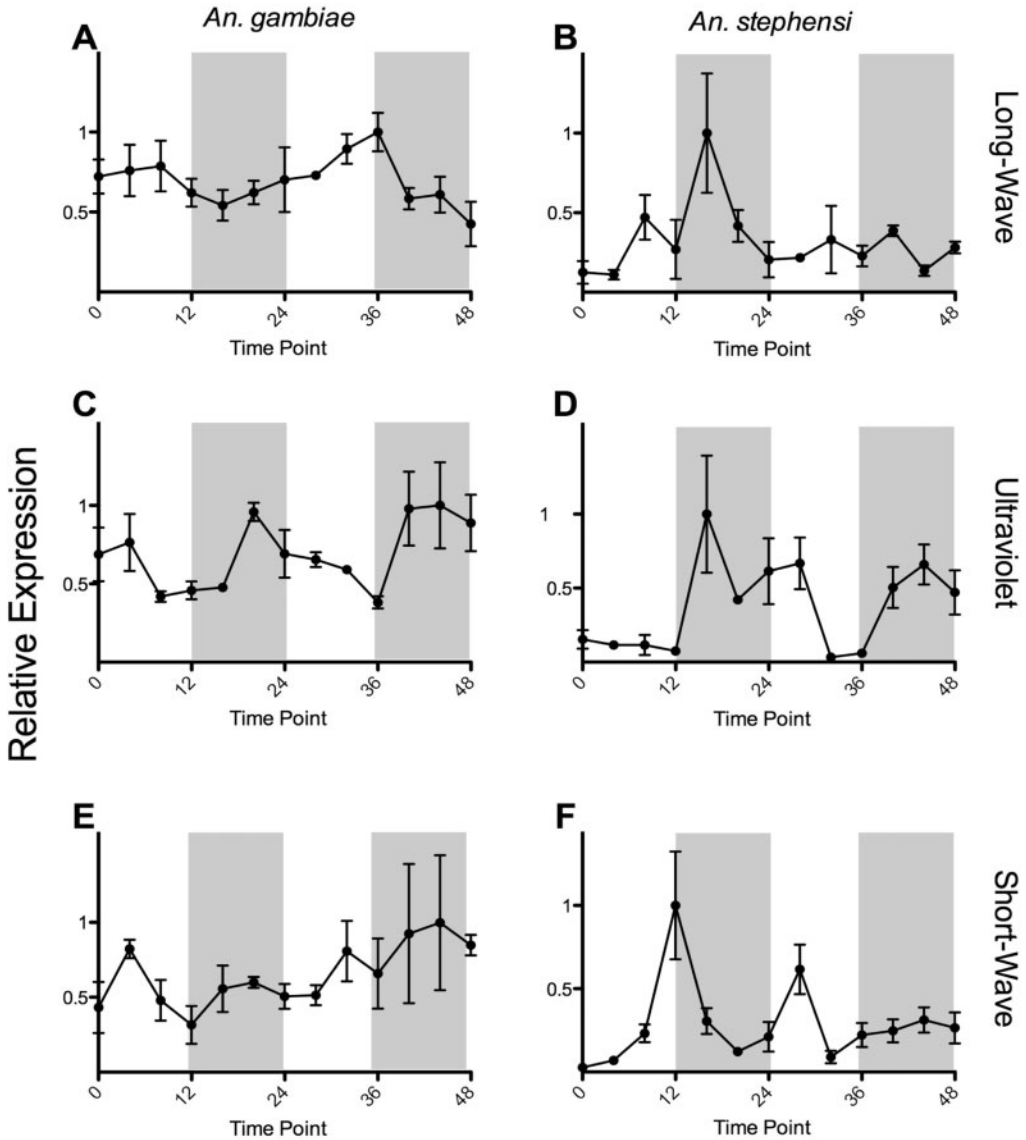

Fig. 4. Opsin expression profiles across Zeitgeber time. Relative quantity ($2^{\Delta Ct} \pm$ SEM) of *opsin* gene transcripts normalized to *ribosomal protein subunit-7* transcript, respectively. Time points indicate samples taken every 4 h, with time point 0 being at the beginning of a 11:11 light:dark cycle with 1 h dusk:dawn transition periods, spanning two full diurnal cycles. Each time point consists of collections of 10 female mosquitos, with $N = 3$. Values are normalized so the highest level of expression is equal to one for each analysis. Filled bars represent time points sampled during the dark phase of the cycle. Open bars represent time points sampled during the light phase of the cycle. Panels A,D and E represent *Anopheles gambiae* long-wave (GPROP3), ultraviolet (GPROP8) and short-wave (GPROP9) gene levels, respectively. Panels B, C and F represent putative orthologous long-wave, ultraviolet and short-wave genes in *Anopheles stephensi*.

GPROP10, an opsin of unknown wavelength sensitivity, is expressed predominantly during adult stages. The remaining *opsin* genes—*GPROP8* and *GPROP9*—which encode one ultraviolet wavelength-sensing opsin and one short wavelength-sensing opsin, respectively, each exhibit higher expression in adults as compared with first and third instar larvae.

The developmental partitioning of *opsin* superfamily gene expression that we observe—most notably the dichotomous expression of long wavelength-sensing *opsin* genes between larval and adult stages—is unexpected and may have functional implications. Past studies of *opsin* gene expression during *An. gambiae* development have utilized the Plasmodium/Anopheles Genome Array, which groups long wavelength-sensing *GPROP1*, *GPROP3*, and *GPROP4* genes into a single probe set (Ag.2R.268.0_CDS_s_at from VectorBase; Marinotti et al. 2006, Rund et al. 2011). Thus, the respective expression profiles for these three genes have not been defined previously. Each of the other

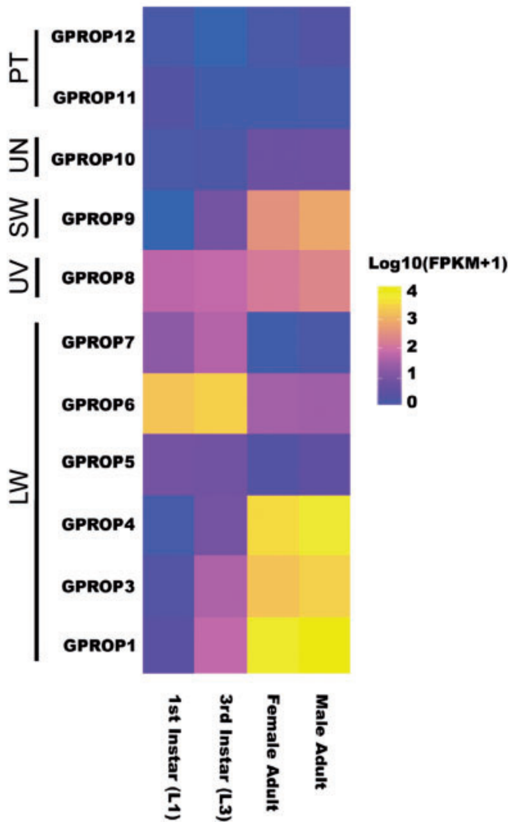

Fig. 5. Heatmap of *An. gambiae Opsin* gene expression. Expression of *Opsin1, 3-12* in *An. gambiae* in mixed-gender first larval instars (L1), mixed-gender third larval instars (L3), adult females (FB), and adult males (MB). Color intensity scale indicates increasing expression, with yellow reflecting the highest expression, measured as FPKM, and blue reflecting the lowest expression. VectorBase ID identifiers and names are given for each transcript. All *opsin* genes are also grouped based on wavelength detected, PT (pteropsin), UN (unknown), SW (short wavelength), UV (ultraviolet wavelength), LW (long wavelength).

long wavelength-sensing *opsin* genes (*GPROP5*, *GPROP6*, and *GPROP7*) is detectable with distinct probes on the array, respectively, allowing for accurate expression profiling of those three *opsin* genes. The use of RNAseq has allowed us to define the expression of each of these *opsin* genes, despite the very limited sequence variation among them, and its use will enable delineation of these paralogs in subsequent analyses.

The fact that half of long wavelength-sensing *opsin* genes are expressed predominantly during larval stages implies that these opsins may mediate functions specific to larval life stages. In this regard, it is notable that gene structures for the subset of long-wavelength sensing *opsin* genes expressed predominantly during larval stages exhibit structural similarities that distinguish them from those expressed predominantly in adults (Fig. 3). Larval-biased *GPROP5*, *GPROP6*, and GRP*OP7* genes each include two exonic CDS regions, and significant 5′ UTR and 3′ UTR regions are present in *GPROP5* and *GPROP6*. In contrast, adult-biased *GPROP1*, *GPROP3*, and GPRO*P4* each contain a single splice-site within the 5′-UTR of each gene and minimal 3′ UTRs and the entireties of their coding capacities reside within a single exon, respectively. These differing structures are consistent with the hypothesis that the two stage-biased *opsin* gene subsets arose from duplication of distinct ancestral genes, with limited subsequent divergence of coding sequences and gene organization within each subset.

However, the life stage-biased functions these long wavelength-sensing opsins mediate remain unclear. Visual acuity may play an important role during larval life stages for the detection of predators within aqueous environments (Klecka and Boukal 2012), while adults may process figures or shapes from the air in search of potential sugar sources, bloodmeal sources, resting sites, and oviposition sites (Allan et al. 1987). The predominant expression of some long wavelength-sensing *opsin* genes during larval stages, and the expression of other long wavelength-sensing *opsin* genes, and short wavelength-sensing and ultraviolet wavelength-sensing *opsin* genes only in adults may have arisen because of differing opsin requirements underlying visual acuity in aqueous environments as compared with atmospheric environments.

Subsets of long wavelength-sensing opsins are arranged in homologous loci, which are partially conserved between *An. gambiae* and *An. stephensi* (Fig. 3). The homologous locus in *An. gambiae* that contains two larval-biased genes and one adult-biased gene (i.e., *GPROP4-6*) is highly conserved in *An. stephensi*. If these gene trios are derived from a single gene cluster in the most recent common ancestor (MRCA) of *An. gambiae* and *An. stephensi*, then that MRCA may have possessed similar larval–adult variability in the expression of long wavelength-sensing *opsin* genes. Similarly, *An. stephensi* contains an ortholog of *An. gambiae GPROP7*, and genomic regions surrounding the orthologous gene in each species appear to be syntenic as reflected by the location of *An. gambiae* and *An. stephensi GPROP7* orthologs next to *AGAP002463* and *ASTE008930*, respectively, which are orthologs with homologies to ubiquitin-associated and SH3 domain-containing protein B (UBASH3B [Megy et al. 2012], Fig. 3). Taken together, these observations imply that the *GPROP4-6* long wavelength-sensing *opsin* gene cluster and the *GPROP7* orthologs were present in the MRCA of these two species. This invites the hypothesis that the gene family expansion in *An. gambiae* that created *GPROP1* and *GPROP3* occurred after divergence of the two species, and that the differing illumination preferences in the two species also arose following their divergence from a common ancestor, in conjunction with *opsin* gene family expansion. As *GPROP1* and *GPROP3* are expressed predominantly in adults, *An. gambiae* may have been selected during its evolutionary history for greater photosensitivity based on a mechanism mediated by adult *opsin* gene expression. Other organisms, such as butterflies, that exhibit increases in long wavelength-sensing *opsin* gene number also exhibit expanded

spectral diversity for visual function (Sison-Mangus et al. 2006, Frentiu et al. 2007). Therefore, the expansion of long wavelength-sensing *opsin* gene number may underlie dynamic evolution of visual sensitivity across an expanded spectral range in *An. gambiae*, as compared with *An. stephensi*.

In conclusion, we have begun to investigate molecular mechanisms underlying photopreference differences we observe between *An. gambiae* and *An. stephensi* in laboratory-based assays, by assessing differences between these two species in the genomic organization and expression of *opsin* genes. Using binary and trinary choice assays, we determine that *An. gambiae* and *An. stephensi* prefer different illumination intensities at subjective dawn and dusk. Analyses of long wavelength-, short wavelength- and ultraviolet wavelength-sensing *opsin* gene expression, measured over two diurnal cycles, reveal no conclusive differences between these two species in diurnal patterns of transcript expression. RNAseq data sets in *An. gambiae* indicate that long wavelength-sensing *opsin* genes are expressed in two distinct gene sets, during either larval or adult stages. Conversely, short wavelength- and ultraviolet wavelength-sensing *opsin* genes are expressed more highly in adult females and males. *An. stephensi* possess only four long wavelength-sensing *opsin* genes compared with the six long wavelength-sensing *opsin* genes found in *An. gambiae*, a reflection of paralogous expansion of the *opsin* gene superfamily in *An. gambiae*. The differences we observe in the number of long wavelength-sensing *opsin* genes in these two species may contribute to the differences we observe between their illumination intensity preferences.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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