Quantitative real-time PCR analysis of *Anopheles dirus TEP1* and *NOS* during *Plasmodium berghei* infection, using three reference genes

Jonathan W.K. Liew, Mun Yik Fong and Yee Ling Lau

Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

ABSTRACT

Quantitative reverse transcription PCR (qRT-PCR) has been an integral part of characterizing the immunity of *Anopheles* mosquitoes towards *Plasmodium* invasion. Two anti-*Plasmodium* factors of *Anopheles*, thioester-containing protein 1 (TEP1) and nitric oxide synthase (NOS), play a role in the refractoriness of *Anopheles* towards *Plasmodium* infection and are generally expressed during infection. However, these are less studied in *Anopheles dirus*, a dominant malaria vector in Southeast Asia. Furthermore, most studies used a single reference gene for normalization during gene expression analysis without proper validation. This may lead to erroneous quantification of expression levels. Therefore, the present study characterized and investigated the expression profiles of *TEP1* and *NOS* of *Anopheles dirus* during *P. berghei* infection. Prior to that, the *elongation factor 1-alpha* (*EF1*), *actin 1* (*Act*) and *ribosomal protein S7* (*S7*) genes were validated for their suitability as a set of reference genes. *TEP1* and *NOS* expressions in *An. dirus* were found to be significantly induced after *P. berghei* infection.

INTRODUCTION

Advances in molecular biology and gene expression techniques have enabled researchers to identify genes involved in the immunological response of *Anopheles* mosquitoes towards *Plasmodium* infection (*Chen, Mathur & James, 2008*; *Volohonsky et al., 2017*). Understanding of this immunity complex may lead to development of novel approaches for controlling malaria transmission, such as transgenic mosquitoes and inhibition of parasite development in the mosquito. The immunological changes caused by *Plasmodium* infection at the transcriptome level can be captured in microarray gene expression and quantitative reverse transcription PCR (qRT-PCR) analyses (*Aguilar et al., 2005*). The qRT-PCR is used as a tool to validate expression data from microarray analyses and to detect changes in the expression levels of target genes.

Thioester-containing protein 1 (TEP1) has been shown to mediate anti-*Plasmodium* responses in various mosquito species (*Clayton, Dong & Dimopoulos, 2014*; *Jaramillo-Gutierrez et al., 2009*). In the hemolymph of the mosquito, TEP1, leucine-rich repeat immune protein 1 (LRIM1) and *Anopheles-Plasmodium*-responsive leucine-rich repeat 1

Corresponding author
Yee Ling Lau, lauyeeling@um.edu.my

(APL1) protein combine to form a stable complex (*Fraiture et al., 2009*; *Povelones et al., 2011*). The precise mode of action of this TEP1-associated complex is not known but it has been implicated in the vector competence of *An. gambiae* (*Blandin et al., 2004*; *Fraiture et al., 2009*) and *An. quadrinnulatus* (*Habtewold et al., 2008*). Silencing of the TEP1, LRIM1 or APL1 gene abolishes parasite melanization and converts refractory or non-compatible *Anopheles* strain into a susceptible one.

Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline in the mosquito, producing nitric oxide which is toxic to the parasite (*Clayton, Dong & Dimopoulos, 2014*). The generated nitric oxide in the midgut cells and subsequent tyrosine nitration kill the parasite ookinetes as they traverse the midgut epithelial cells (*Kumar et al., 2004*). Furthermore, NOS plays a role in hemocyte-mediated immune responses in the hemocoel of the mosquito and is expressed in the hemocytes and fat body (*Hillyer & Estevez-Lao, 2010*). Elevated levels of *NOS* gene expression have been observed in the midgut epithelial cells of *An. stephensi* invaded by *P. berghei* ookinetes (*Han et al., 2000*). Moreover, chemical inhibition of NOS resulted in an increase in the number of oocysts in infected *An. stephensi* (*Luckhart et al., 1998*) and *An. culicifacies* B (*Vijay et al., 2011*). These findings indicate that NOS is also a determinant of vector refractoriness in *Anopheles* mosquitoes (*Vijay et al., 2011*). Interestingly, a study has shown that some NOS activity is required for survival of the *Plasmodium* at the early developmental stages but it has an opposite effect at a later stage by limiting oocyst survival in the mosquito (*Gupta et al., 2009*).

The TEP1 and NOS may be working in concert to eliminate parasites in the mosquito. The heme peroxidase (HPX2)/NADPH oxidase 5 (NOX5) system of *An. gambiae*, along with NOS, mediates nitration of the midgut epithelial cells and potentiates nitric oxide toxicity against *P. berghei*. Evidence shows that epithelial nitration precedes TEP1-mediated lysis. *De Almeida Oliveira, Lieberman & Barillas-Mury (2012)* proposed that epithelial nitration is needed for effective TEP1-mediated lysis of ookinetes. Furthermore, silencing the TEP1, leucine-rich repeat proteins and HPX2/NOX5, renders the resistant *An. gambiae* L3-5 strain susceptible to *P. berghei* infection. These findings demonstrate the concerted roles of the proteins in the defense mechanism of *Anopheles* mosquitoes (*Eldering et al., 2016*).

It is evident that these studies have greatly advanced our understanding of *Anopheles* immunity and qRT-PCR has been featured as an important investigative tool in these informative studies. However, most of the investigations used only one reference gene (often, the *ribosomal protein S7* gene) for normalization of expression data and no validation was documented. As robust as qRT-PCR is, it may still be subjected to systematic error from technical and biological limitations (*Kozera & Rapacz, 2013*; *Omondi et al., 2015*). Thus, proper normalization is crucial to ensure an accurate result. It is recommended that at least three different reference genes be used for normalization as this will increase accuracy and resolution. Furthermore, the reference genes for normalization should be validated for different experimental designs to obtain reliable gene expression results (*Bustin et al., 2009*; *Derveaux, Vandesompele & Hellemans, 2010*; *Ponton et al., 2011*).

Anopheles dirus is the primary malaria vector in Southeast Asia and is capable of transmitting all human malaria parasites (*Coatney et al., 1971*; *Manguin et al., 2008*; *Marchand et al., 2011*; *Nakazawa et al., 2009*; *Vythilingam et al., 2005*). However, molecular

understanding of immune responses in *An. dirus* is still lacking. The recent availability of the *An. dirus* genome sequence (*Neafsey et al., 2015*) should facilitate and enhance research on its immune response mechanisms. Given the important roles TEP1 and NOS play in the immune defense of *An. gambiae* and *An. stephensi* against *Plasmodium*, we postulate that both immune factors in *An. dirus* would play similar roles against the parasites.

Thus, the current study aims to have better understanding of the molecular immune responses in an important Southeast Asian malaria vector. The implementation of a proper qRT-PCR assay is also important to yield data that is more comparable and reliable. Indeed, these will advance the characterization of immune traits and transmission mechanisms of major malaria vectors in a local context. Aside from a few of studies reporting the use of the *An. dirus/P. berghei* model (*Fracisco et al., 2010*; *Lapcharoen et al., 2012*), *An. dirus* has been determined to be permissive to *P. berghei* infection in the laboratory (JWK Liew, 2017, unpublished data). Hence, the current study validated elongation factor 1-alpha (*EF1*), actin 1 (*Act*) and *S7* as reference genes for normalization in qRT-PCR, followed by investigation on *TEP1* and *NOS* expression profiles in *An. dirus* during *P. berghei* ANKA infection.

MATERIALS AND METHODS

Total RNA extraction of whole mosquitoes and cDNA synthesis

Total RNA was extracted from pooled whole mosquitoes as previously described (*Khan et al., 2016*). Each batch of RNA was extracted from three female mosquitoes using ReliaPrep™ RNA Tissue Miniprep System (Promega, Madison, WI, USA). The pooled mosquitoes were first cold-anesthetized before homogenization in cold lysis buffer using the handheld homogenizer, BioMasher-II (Nippi, Tokyo, Japan). The total RNA was then rendered DNA-free using TURBO DNA-*free*™ kit (Ambion, Vilnius, Lithuania). cDNA was synthesized from 150 ng of total RNA using the qPCRBIO cDNA synthesis kit (PCR Biosystems, London, UK).

PCR amplification of *AdTEP1* and *AdNOS* coding sequences and of *AdEF1* and *AdAct* partial sequences

At the time of this study, the *An. dirus* genome was not sequenced. Only *An. dirus* TEP1 (GenBank: FJ263422) and *S7* (GenBank: AY369135.2) partial sequences were available. Thus, rapid amplification of cDNA ends (RACE), using the SMARTer™ RACE cDNA Amplification kit (Clontech, California, USA) was performed to obtain full length coding sequences of *AdTEP1* and *AdNOS*. Nested RACE PCR was carried out according to the manufacturer's instructions. For elongation factor 1-alpha, *AdEF1* was amplified using degenerate primers designed based on aligned sequences of *Aedes aegypti* (GenBank: DQ440206), *Culex quinquefasciatus* (GenBank: XM_001850793) and *An. gambiae* (GenBank: XM_308429.3), while partial sequence of *AdAct* was amplified using universal primers designed by *Staley et al. (2010)*. These PCR amplifications were performed using DreamTaq Green DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). All amplicons were cloned into pGEM®-T vector (Promega, Madison, USA) and sequenced. The primers used for both PCR are shown in Table 1.
Table 1 Primers used in PCR.

Primer	Sequence (5′–3′)
TEP1	
GSP1: Ad5RaTEP1	GTCCTAGAACCCTGATGCTCCAGCAGTGC
NGSP1: Ad5RaTEP1N1	CGATGTCAGCGCTACACCATTCCGCAGACC
GSP2: Ad3RaTEP1	CAAGCAGACGGCTCCTTCGGTGTGTGG
NGSP2: Ad3RaTEP1N2	GCTGGTTGAGAGGGCATACGAGTGGCTCG
NOS	
GSP1: AdNOS5Ra	CCTCGCGCGACAGTGCGAGGAACACCCG
NGSP1: Ad5RaNOSN1	CTGGACCATCTCCTGCTTCTCGTCCCGG
GSP2: AdNOS3Ra	GTGCGCAGCGCACCGTCGTTCCACATGTCG
NGSP2: Ad3RaNOSN2	CCGACCAAGCCGGTCATCCTGATCGGTC
EF1	
EF1-F1	ATGGGTAAGGARAAGACTCA
EF1-R1	GACCTTCTCCTTGATYTCG
Act	
Act-2F	ATGGTCGGYATGGGNCAGAAGGACTC
Act-8R	GATTCCATACCCAGGAAGGADGG

Molecular and phylogenetic analysis of *AdTEP1* and *AdNOS*

The *AdTEP1* and *AdNOS* consensus sequences were formed from the 5′-RACE and
3′-RACE PCR products of each gene using BioEdit Sequence Alignment Editor v7.2.3.
BLAST (*Johnson et al., 2008*) and Open Reading Frame Finder (ORFfinder) programs from
NCBI were used to obtain and analyze the coding sequences of *AdTEP1* and *AdNOS*.
Putative conserved domains and protein structure were analyzed using BLASTP. The TEP1
and NOS sequences of several insects were aligned using Clustal Omega and phylogenetic
trees were constructed (Jukes-Cantor model; neighbor-joining method; 1,000 bootstrap
value) using MEGA 7.0.21 software. All sequences were submitted to GenBank.

Plasmodium berghei infection of *An. dirus* mosquitoes

Anopheles dirus WRAIR2 strain was reared at a temperature of 25–27 °C, 70–80% humidity
and 12:12 h light-dark photoperiod. In every experimental replicate, five- to seven-day old
female *An. dirus* mosquitoes, emerged from the same larva tray were used. The mosquitoes
were placed in the same cage and given 10% sugar solution supplemented with Vitamin
B complex, *ad libitum*. One night before blood feeding on a *P. berghei* ANKA-infected
BALB/c female mouse, female mosquitoes were starved in an ambient temperature of
20 ± 1 °C and 80% humidity. Subsequently, blood feeding and containment of engorged
mosquitoes were performed in the same environmental conditions. Female mosquitoes
fed on healthy mouse were used as controls. All mosquito infections were performed at
gametocytemia of 0.42–0.64% (parasitemia: 5.86–6.36%). It was found that fully engorged
mosquitoes which fed on such levels of parasites, will have a substantial number of oocysts
in their midguts and are 100% prevalent with sporozoites present in their salivary glands

Table 2   Primers used in qRT-PCR.

Primer	Sequence (5′–3′)	Expected size (bp)	PCR efficiency
TEP1			
AdrtTEP1F3	GGCAAAGTCCATGCAAAC	126	103.3
AdrtTEP1R3	ATAACGGAACCAACCTCATC		
NOS			
AdrtNOSF2	GGAGAAAGCGCACATCTAC	116	109.2
AdrtNOSR2	ACTTCTCCATTTCCGTTCC		
EF1			
AdrtEF1F1	CCGGACATCGTGATTTCAT	118	102.8
AdrtEF1R1	TGGCCGTTCTTGGAGATA		
Act			
AdrtACTF2	TCTGACCGACTACCTGAT	130	100.0
AdrtACTR2	CATCTCCTGCTCGAAGTC		
S7			
AdrtS7F1	GAGGTCGAGTTCAACAACAA	132	108.4
AdrtS7R1	GAACACGACGTGCTTACC		

(JWK Liew, 2017, unpublished data). Ethical approval for the study was obtained from the Faculty of Medicine Institutional Animal Care and Use Committee, University of Malaya, Malaysia (Ethics Reference no.: 20150407/PARA/R/MBK).

Quantitative reverse transcription PCR of *AdTEP1* and *AdNOS* post *P. berghei* infection

Total RNA was extracted at 12 h, 24 h, 48 h and Day 5 post infection (PI) and reverse transcribed. The PCR conditions were the same for all primers (Table 2) i.e., 95 °C for 30 s, 40 cycles of 95 °C for 10 s and 57.6 °C for 25 s; and a melt curve step from 65 to 95 °C, with increment of 0.5 °C every 5 s. The qPCR was performed using SsoAdvanced[TM] Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) on Bio-Rad CFX96[TM] Real-Time System. The primers were designed using the online PrimerQuest tool on Integrated DNA Technologies website, following the suggestions in the SsoAdvanced[TM] Universal SYBR® Green Supermix instruction manual. A 20 µL reaction contained 312.5 nM of each forward and reverse primer and 6 ng of cDNA. Results of the PCR were analyzed by the Bio-Rad CFX Manager[TM] 3.1 software. Expression levels were normalized against those of *EF1*, *Act* and *S7*. Relative expression was calculated using the $2^{-\Delta\Delta C_T}$ formula, compared to the controls. The experiment was repeated 4 times.

Gene expression stabilities and statistical analysis

To determine the gene expression stabilities of *AdEF1*, *AdAct* and *AdS7*, NormFinder v0.953 Microsoft Excel add-in (*Andersen, Jensen & Orntoft, 2004*) and Bio-Rad CFX Manager[TM] 3.1 software were used. The CFX manager software determines the target stability values by adopting the pairwise variation strategy as in geNorm (*Vandesompele et al., 2002*). Additionally, the comparative delta-Ct method (*Silver et al., 2006*) was also employed to
Table 3 Coding DNA region length and protein length of genes sequenced in this study.

Gene	Total base pairs (bp)	Protein length (amino acids)	GenBank accession no.	Isoelectric point[a]	Molecular weight (Dalton)[a]
TEP1	2,733	910	KY465474	6.91	102644.20
NOS	3,006	1,001	KY465473	6.22	114212.80
EF1	1,137	–	KY022437	–	–
Act	683	–	KY022436	–	–

Notes.
[a]Predicted by ExPASy Compute pI/Mw tool (*Gasteiger et al., 2003*).

establish gene stability. For statistical analysis, one-way ANOVA with multiple comparisons of Tukey was performed with 95% confidence intervals (alpha = 0.05) using GraphPad Prism version 5.01.

RESULTS

Molecular and phylogenetic analyses of *AdTEP1* and *AdNOS* proteins

Details of the proteins are shown in Table 3. The *AdTEP1* coding sequence has 71% and 98% similarity to *An. gambiae TEP-1* mRNA (GenBank: AF291654.1) and *An. dirus* strain WRAIR2 contig 1.4921, whole genome shotgun (WGS) sequence (GenBank: APCL01004922.1), respectively. Putative conserved domains detected on the protein include alpha-2 macroglobulin associated components (A2M-N-2, A2M receptor, A2M complement component), thioester regions and isoprene C2-like family (Fig. 1). As for *AdNOS*, it is shown to be 89% similar to *An. gambiae* str. PEST AGAP008255-PA mRNA (GenBank: XM_317213.1) and 99% similar to *An. dirus* strain WRAIR2 contig 1.1607, WGS sequence (GenBank: APCL01001608.1). BLASTP detected typical conserved domains of NOS proteins in the protein sequence (Fig. 1).

The NOS protein is typically conserved among insect species, demonstrating high sequence similarity, especially among the anopheline mosquitoes. On the other hand, the insects' TEP1 protein sequences are considerably conserved, but do not exhibit high sequence identity as that of NOS protein (Table 4). Multiple sequence alignment (File S1) showed that the TEP1 protein sequences vary among the insects, albeit are more similar within the *Anopheles* genus. *AdTEP1* is closer in homology to that of *An. gambiae* and *An. arabiensis*. This is also reflected in the phylogenetic tree (Fig. 2).

EF1, *Act* and *S7* are suitable reference genes for the study

The suitability of *EF1*, *Act* and *S7* as reference genes for gene expression analysis of *P. berghei*-infected *An. dirus* was addressed under the experimental conditions of this study, in conjunction with the SYBR green intercalating dye chemistry. The stability values of the three genes, as analyzed by the Bio-Rad CFX Manager software showed that they were stably expressed as the values were in the acceptable range. When all three genes were collectively used across 16 separate qRT-PCR reactions (4 time points × 4 replicates), the average mean coefficient of variance (CV) was 0.109 (SD: 0.053) while the average mean
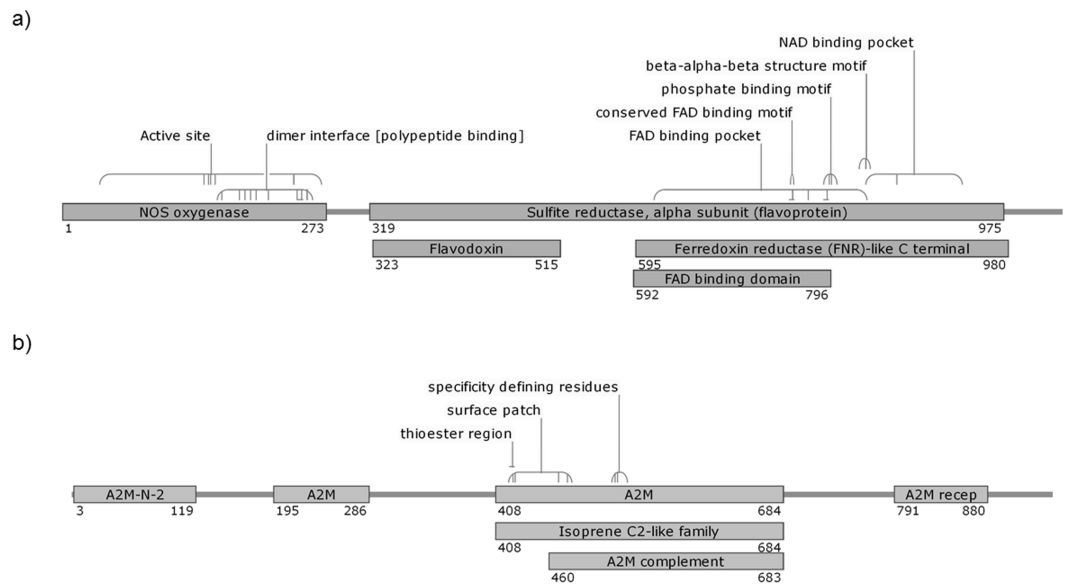
Figure 1 Putative conserved domains of *An. dirus* (A) NOS and (B) TEP1 protein detected by BLASTP. The numbers denote the region of the domains on the protein sequence. The diagrams are not drawn to scale. FAD, flavine adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; A2M-N-2, Alpha-2-macroglobulin family N-terminal region; A2M, Protein similar to Alpha-2-macroglobulin; A2M recep, Alpha-2-macroglobulin receptor; A2M complement, Alpha-2-macroglobulin complement component.

Table 4 Percentage similarity of *AdTEP1* and *AdNOS* proteins to those of other organisms, calculated by Clustal Omega, available at the EMBL-EBI website (*Sievers et al., 2011*).

Organism	GenBank accession no.	% Identity
TEP1		
An. gambiae	AAG00600	72.28
An. arabiensis	ACG68535	72.28
An. sinensis	KFB36250	61.67
An. darlingi	ETN59728	54.71
Ae. aegypti	XP_001660377	42.54
Cx. quinquefasciatus	XP_001842016	41.48
D. melanogaster	NP_523578	28.17
NOS		
An. stephensi	O61608.2	97.60
An. gambiae	XP_317213	96.20
An. sinensis	KFB45232	95.10
An. darlingi	ETN60994	85.47
An. aquasalis	AEK26396	78.21
Ae. aegypti	XP_001660328	88.50
Cx. quinquefasciatus	XP_001842036	75.88
D. melanogaster	NP_001027243.2	73.75
a)

b)
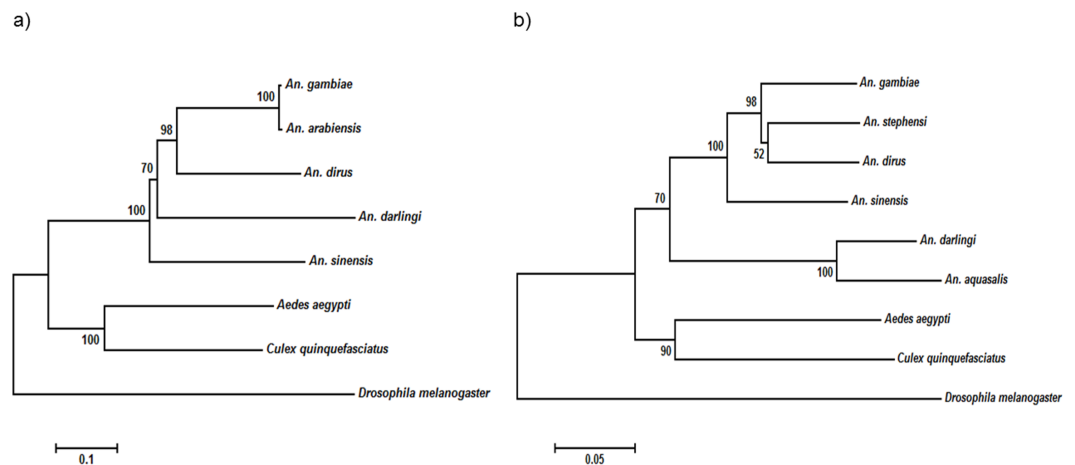
Figure 2 Phylogenetic tree of (A) *TEP1* gene and (B) *NOS* gene. The scale bar at the bottom are in the units of number of base substitutions per site.

M value was 0.271 (SD: 0.133). Stably expressed reference genes of homogeneous samples should exhibit a mean CV of <0.25 and expression stability, *M* value of <0.5 (*Hellemans et al., 2007*).

NormFinder identified *Act* as the best reference gene and *Act/S7* (stability value: 0.040) as the best combination when comparing normal blood fed and infected groups. Whereas, in experiments comparing between timepoints, *Act* was also the best gene but the best combination of two genes was *EF1/Act* (stability value: 0.095). Using the comparative delta-Ct method, the best combinations in descending order were *Act/S7*, *EF1/Act*, *EF1/S7* (Fig. 3). geNORM also identified combination *Act/S7* (*M* value: 0.023) as the best, followed by *EF1/Act* (*M* value: 0.028) and *EF1/S7* (*M* value: 0.041). Furthermore, the relative expressions of *AdTEP1* and *AdNOS* were compared when a single or combination of reference genes were used for normalization (Table 5). Variation could be seen when expression was normalized against a single reference gene. Nevertheless, to adhere to the MIQE guidelines (*Bustin et al., 2009*), these three reference genes were used together in the current gene expression study.

AdTEP1 and *AdNOS* are transcriptionally induced during *P. berghei* infection

The gene expressions of *AdTEP1* and *AdNOS* at different intervals (12 h, 24 h, 48 h and Day 5) PI were studied in quadruplicates. The time points were chosen to coincide with developmental stages of the parasite: 12 h—ookinete; 24 h—early infected stage; 48 h—early oocyst; Day 5—intermediate oocyst stage (*Xu et al., 2005*). The control group consisted of mosquitoes which took a normal blood meal. The relative expressions of these genes are shown in Fig. 4. Transcription of *AdNOS* gene peaked to about 2-fold at 48 h PI and returned to basal level at Day 5 PI. Whereas for *AdTEP1*, there was an approximately 3-fold increase in expression 24 h PI. The upregulation of *AdNOS* and *AdTEP1* was significant but other than their respective peaks at 48 h and 24 h PI, the genes exhibited almost basal expression at all other time points.
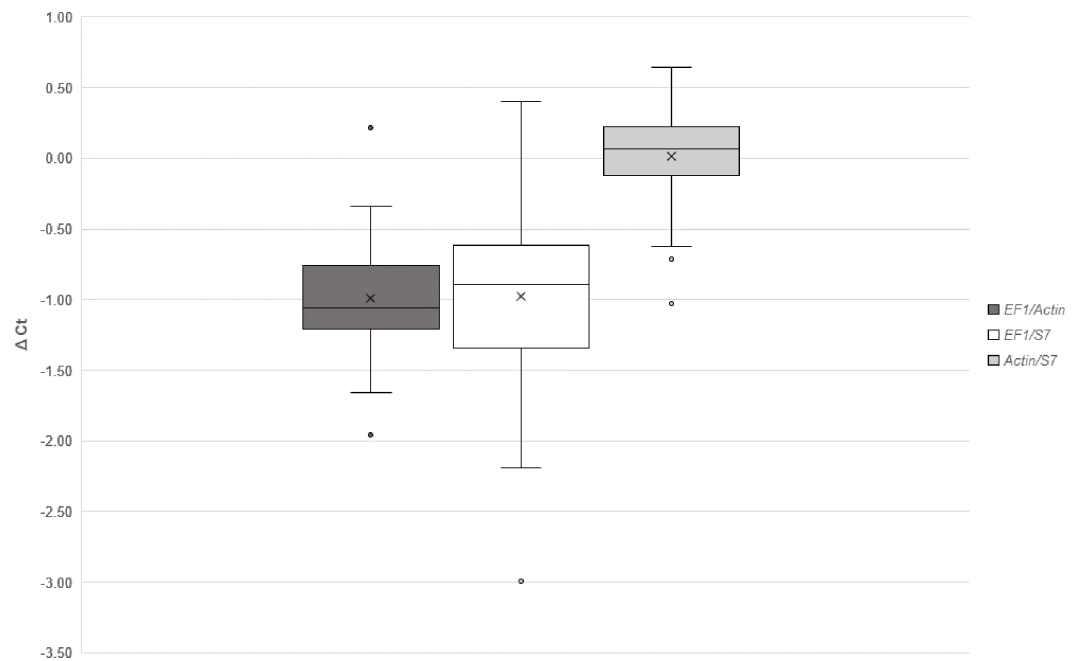
Figure 3 Comparative ΔCt method for selection of reference genes.

Table 5 Normalized relative expressions of *AdTEP1* and *AdNOS* at 24 h and 48 h PI using different combination of reference gene(s).

Gene	Reference gene	24 h PI	48 h PI
TEP1	EF1	3.533	1.091
	Act	2.685	1.499
	S7	3.187	1.435
	Act/S7	2.925	1.467
	EF1/Act	3.08	1.278
	EF1/S7	3.356	1.251
	EF1/Act/S7	3.115	1.329
NOS	EF1	1.305	1.446
	Act	0.992	1.987
	S7	1.178	1.903
	Act/S7	1.081	1.945
	EF1/Act	1.138	1.695
	EF1/S7	1.240	1.659
	EF1/Act/S7	1.151	1.762

DISCUSSION

AdTEP1 and *AdNOS* proteins are homologous with those of other *Anopheles*. Structurally, *AdNOS* protein possesses the N-terminal oxygenase domain which contains a putative heme; calmodulin which links the N-terminal and C-terminal; and the C-terminal FAD/NADPH cofactor-binding domain. These have been reported to be evolutionarily
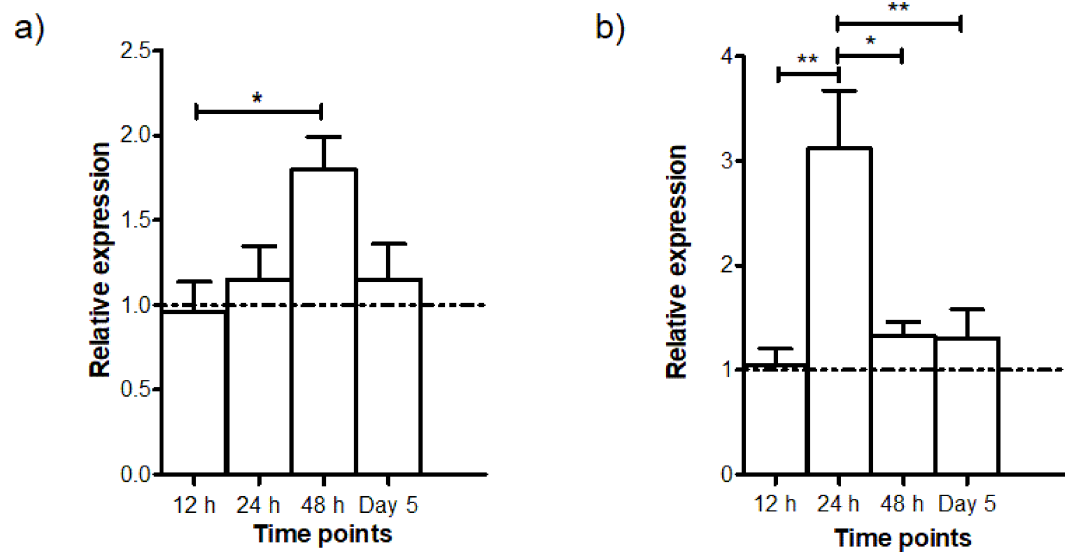
Figure 4 Time-point gene expression post infection (mean ± SEM) of (A) *AdNOS* and (B) *AdTEP1* in *P. berghei* ANKA-infected *An. dirus.* The dotted lines at the relative expression axes indicate the expression levels of the control group (normal blood meal). *significant (0.01 < p < 0.05); **very significant (0.001 < p < 0.01).

conserved (*Luckhart & Rosenberg, 1999*). There are in fact, multiple transcripts of *NOS* in *An. stephensi* from a single copy of the gene, suggesting alternative splicing and alternative initiation events (*Luckhart & Li, 2001*). Investigation is underway to determine if this is also the case for *An. dirus*. On the other hand, TEP1 is structurally homologous to human complement factor C3 and contains an α-helical thioester region (*Baxter et al., 2007*). It is secreted as a full-length protein and is proteolytically cleaved to produce mature and active TEP1 (*Volohonsky et al., 2017*). As principal components of immunity, both *NOS* and *TEP1* genes are under selective pressure which leads to polymorphism (*Le et al., 2012*; *Luckhart & Rosenberg, 1999*). Polymorphism at the *TEP1* locus gave rise to the *Plasmodium* refractory (R) and susceptible (S) strains of *An. gambiae*. R strain is homozygous for *TEP1*R1* allele, while S strains contain *TEP1*S1/S2/R2* alleles (*Eldering et al., 2016*). The TEP1 alleles segregate in wild *An. gambiae* and *An. arabiensis* populations (*White et al., 2011*). It is unknown if this is also the case for *An. dirus*.

There are no universal reference genes or normalization method for qRT-PCR (*Hellemans et al., 2007*; *Ponton et al., 2011*). At least three reference genes have to be validated for their stabilities under different experimental conditions (*Bustin et al., 2009*; *Derveaux, Vandesompele & Hellemans, 2010*). *Anopheles* ribosomal protein *S7* is routinely used as a reference gene in studies similar to this. There are also gene expression studies of *Anopheles* using *actin* (*Li et al., 2014*; *Luckhart et al., 1998*) or *EF1* (*Omondi, Majeed & Ignell, 2015*) as a reference gene. However, most of these investigations utilized only a single reference gene for normalization, which is not recommended (*Bustin et al., 2009*; *Vandesompele et al., 2002*). Moreover, expression of *S7* of *An. stephensi* was significantly influenced by different temperature treatments (*Murdock et al., 2012*). Thus, total RNA

concentration was included as control for background expression levels in that study. Elongation factor 1-alpha expression also fluctuates under temperature stress in the diamondback moth, *Plutella xylostella*, which is otherwise stably expressed (*Fu et al., 2013*). Hence, proper validation of reference genes for qRT-PCR normalization is crucial for accurate result. The three reference genes in this study were selected for validation based on previous studies. Although more candidate genes of different functional classes should be assessed (*Vandesompele et al., 2002*), results showed that *EF1*, *Act* and *S7* were an appropriate set of reference genes for gene expression analysis of *P. berghei*-infected *An. dirus* in this study. Since mosquitoes used in this study were of similar age, it is unlikely that the validities are confounded by ageing. Furthermore *EF1*, *Act* and *S7* have been reported to be reliable reference genes for studies on candidate age-grading genes and on different developmental stages in the parasitoid *Dastarcus helophoroides*, butterfly *Bicyclus anynana*, *Aedes aegypti* and *An. gambiae* (*Dzaki et al., 2017*; *Pijpe et al., 2011*; *Wang et al., 2013a*; *Zhang et al., 2016*).

The upregulation in *AdTEP1* expression at 24 h *P. berghei* infection corroborated well with that of another study using the *An. dirus*/*P. yoelii* model (*Wang et al., 2013b*). It is also similar to that of *An. gambiae* during *Plasmodium* infection. In *An. gambiae*, *TEP1* is constitutively expressed prior infection and is upregulated by 1.8–2.5-fold at 24 h post *P. berghei* infection (*Blandin et al., 2004*; *Gupta et al., 2009*). Then at 48 h PI, the *TEP1* transcript level falls back to as it was before infection (*Frolet et al., 2006*). The same pattern is observed in *An. dirus* (Fig. 4B). In another study, the expression depressed and peaked again at Day 4 PI. The expressions coincide with the traverse of ookinetes through the midgut cells and the development of early oocysts (*Blandin et al., 2004*). Since TEP1 binds to and kills *P. berghei* and *P. falciparum* midgut stages (*Blandin et al., 2004*), it is postulated that *TEP1* expression is induced in the fat body to replenish the protein in the hemolymph after infection (*Gupta et al., 2009*; *Volohonsky et al., 2017*).

The function and regulatory pathway of nitric oxide production in *Anopheles* are evolutionarily conserved and are shared with the vertebrates (*Hillyer & Estevez-Lao, 2010*). This study found that *AdNOS* expression was highest at 48 h PI while having almost basal expression at all other time points. Similarly, *An. stephensi* infected with *P. yoelli* exhibited increased mean NOS expression at 24 h PI which reached peak expression levels at 48 h PI and declined at later stages of oocyst development (*Murdock et al., 2014*). The repression of NOS mRNA levels through nitric oxide feedback reduces NOS protein levels to protect the host from self-induced damage (*Peterson & Luckhart, 2006*). Nitric oxide synthase gene expression in *An. stephensi* appears to follow the development of *P. berghei* or *P. falciparum* in the mosquito. Within 1–3 days post infection, *NOS* was transcriptionally induced coinciding with invasion and early oocyst development, while no induction was recorded on Day 6 PI when oocyst was developing. By Day 9 PI, expression was elevated and persisted till Day 14 possibly when sporozoite penetration of the salivary gland was complete (*Luckhart et al., 1998*). Midgut *NOS* expression was also induced in *An. culicifacies* infected with *P. vivax*, starting Day 1 until Day 15 PI (*Vijay et al., 2011*). In *An. aquasalis* infected with *P. vivax*, *NOS* expression was significantly induced 36 h PI, with NOS protein found mostly in midgut epithelial cells 24 h PI (*Bahia et al., 2011*).

The *Anopheles* immune defenses against *Plasmodium* infections have been extensively studied over the past decades, mostly using the *An. gambiae* and *An. stephensi/P. berghei* and *P. falciparum* infection models (*Cirimotich et al., 2010*; *Clayton, Dong & Dimopoulos, 2014*; *Crompton et al., 2014*). It is believed that there is a broad range of compatibility between different *Plasmodium* and mosquito strains. In addition, infection intensities and different mosquito/*Plasmodium* model influence gene expression and anti-*Plasmodium* responses, except for TEP1 activity that is not affected by infection intensity (*Aguilar et al., 2005*; *Garver et al., 2012*). Therefore, comparisons of results between studies have to be conducted with care. For example, *TEP1* silencing in *An. gambiae* (Keele strain) only doubled the median number of *P. falciparum* NF54 oocysts while *P. berghei* oocysts number increased 4–5-fold (*Jaramillo-Gutierrez et al., 2009*). Silencing the leucine-rich repeat protein genes of *An. gambiae* showed a profound effect on *P. berghei* infection but has no effect on the development of sympatric *P. falciparum* field isolates (*Cohuet et al., 2006*). Furthermore, paralog APL1A is found to be responsible for protection against *P. falciparum* while paralog APL1C is needed to protect against *P. berghei* and *P. yoelii* in *An. gambiae* (*Garver, Dong & Dimopoulos, 2009*; *Mitri et al., 2009*). Thus, distinct immune responses are elicited in response to human and rodent malaria parasite. However, the use of the less compatible *Anopheles/Plasmodium berghei* combination, such as the *An. dirus/P. berghei* model in this study, have contributed immensely to our knowledge on mosquito infection responses (*Aguilar et al., 2005*). Following that, more detailed studies can then be designed to investigate the efficiency and potential of these antiplasmodial responses in natural vector/parasite combinations.

Interpretation of results from this and other infection studies is further complicated by the fact that environmental temperature has significant and diverse effects on mosquito immune responses and vector competence. This temperature factor also forms complex interactions with factors such as nature of immune challenge, diet and time (*Murdock et al., 2012*). This makes comparison of human and rodent malaria infection difficult as their experimental temperatures (*P. berghei*: 19–21 °C; *P. falciparum*: 27 °C), optimum temperature for development and rates of development are different (*Mordecai et al., 2013*; *Paaijmans et al., 2012*). This raise the question of whether the reported disparities in immune responses are, in fact, consequences of parasitic infection, differential temperatures or both. It has been demonstrated that *An. stephensi NOS* expression is significantly affected by sampling time point and temperature. The *NOS* transcript level increased earlier at 24 h post *P. yoelii* infection when mosquitoes were maintained at warmer temperatures of 26–28 °C (*Murdock et al., 2014*). Besides, when *An. stephensi* was exposed to different rearing temperature, *NOS* expression was found to be induced and peaked at a later time point (24 h vs 18 h post exposure) when housed at cooler temperature (18 °C vs 22 °C) (*Murdock et al., 2012*). This may be a limitation of our study as the mosquitoes were placed at 20 ± 1 °C from a rearing temperature of 25–27 °C, 16–18 h prior to blood feeding. Proper studies will need to be planned to investigate and confirm this. Nonetheless, the *AdNOS* gene expression profile in the current study showed that the gene expression was basal at 12 h PI before the upregulation at 48 h PI. Additionally, complex environment drivers such as fluctuation in diurnal temperature, ambient temperature, and time of

infection need to be evaluated for their effects on immunity and resistance of *An. dirus*. It has been shown that, complex interplay of these factors affects *NOS* expression in response to immune challenge (*Murdock, Moller-Jacobs & Thomas, 2013*).

Nevertheless, the current study demonstrates that *An. dirus* express and mount *TEP1* and *NOS* immune responses towards *P. berghei* infection, similar to the other *Anopheles*. Although the use of a rodent malaria parasite model does not necessarily reflect the temperature dependencies of the human malarias and their actual interactions with *Anopheles*, it is useful for preliminary identification and characterization of immune responses in the mosquito (*Jaramillo-Gutierrez et al., 2009*). Interesting findings from initial experiments using *P. berghei* in the laboratory can be subsequently validated with natural *An. dirus/Plasmodium* combinations. Nonetheless, our study concerning the gene expressions of TEP1 and NOS is at a preliminary stage and no protein activity was measured. More functional assays and experiments are underway to further understand the TEP1 and NOS immune responses in *An. dirus*.

CONCLUSION

AdTEP1 and *AdNOS* are homologous among the *Anopheles*. For studies comparing the gene expression of *P. berghei*-infected and normal blood fed *An. dirus*, the genes *EF1*, *Act* and *S7* are appropriate normalization controls in qRT-PCR. Using these validated reference genes, we found that *AdTEP1* and *AdNOS* expressions were highly induced respectively at 24 h and 48 h post *P. berghei* infection. Expression of *TEP1* and *NOS* in response to *Plasmodium* infection is common among the anophelines. The study employed the *An. dirus/P. berghei* model to characterize immune factors that could limit malaria transmission. A more comprehensive look into the *Anopheles* in different parts of the world, will enable better understanding of malaria transmission dynamics and rapid extrapolation of any novel transmission blocking strategies to allopatric *Anopheles* species.

ACKNOWLEDGEMENTS

The following reagents were obtained through BEI Resources, NIAID, NIH: *Anopheles dirus*, Strain WRAIR2, Eggs, MRA-700, contributed by Mark Q. Benedict; and *Plasmodium berghei*, Strain ANKA, MRA-671, contributed by MF Wiser.

Funding

This work was supported by the "Bantuan Kecil Penyelidikan" fund (BKS004-2017) and Postgraduate Research Fund (PG045-2016A) from University of Malaya. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

Bantuan Kecil Penyelidikan: BKS004-2017.
Postgraduate Research Fund: PG045-2016A.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Jonathan W.K. Liew conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Mun Yik Fong and Yee Ling Lau conceived and designed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the paper.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The Faculty of Medicine Institutional Animal Care and Use Committee, University of Malaya, Malaysia, provided full approval for this research (Ethics Reference no. 20150407/PARA/R/MBK).

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The gene sequences are uploaded as File S4. The TEP1 and NOS sequences described here have GenBank accession numbers KY465474 and KY465473, and those of EF1 and Act have the accession numbers KY022437 and KY022436.

Data Availability

The raw data has been uploaded as a Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3577#supplemental-information.

REFERENCES

Aguilar R, Dong Y, Warr E, Dimopoulos G. 2005. *Anopheles* infection responses: laboratory models versus field malaria transmission systems. *Acta Tropica* **95**:285–291 DOI 10.1016/j.actatropica.2005.06.005.

Andersen CL, Jensen JL, Orntoft TF. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research* **64**:5245–5250 DOI 10.1158/0008-5472.CAN-04-0496.

Bahia AC, Kubota MS, Tempone AJ, Araujo HRC, Guedes BAM, Orfano AS, Tadei WP, Rios-Velasquez CM, Han YS, Secundino NFC, Barillas-Mury C, Pimenta PFP, Traub-Cseko YM. 2011. The JAK-STAT pathway controls *Plasmodium vivax* load

in early stages of *Anopheles aquasalis* infection. *PLOS Neglected Tropical Diseases* **5**:e1317 DOI 10.1371/journal.pntd.0001317.

Baxter RHG, Chang C-I, Chelliah Y, Blandin S, Levashina EA, Deisenhofer J. 2007. Structural basis for conserved complement factor-like function in the antimalarial protein TEP1. *Proceedings of the National Academy of Sciences of the United States of America* **104**:11615–11620 DOI 10.1073/pnas.0704967104.

Blandin S, Shiao S-H, Moita LF, Janse CJ, Waters AP, Kafatos FC, Levashina EA. 2004. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell* **116**:661–670 DOI 10.1016/S0092-8674(04)00173-4.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55**:611–622 DOI 10.1373/clinchem.2008.112797.

Chen XG, Mathur G, James AA. 2008. Gene expression studies in mosquitoes. *Advance in Genetics* **64**:19–50 DOI 10.1016/s0065-2660(08)00802-x.

Cirimotich CM, Dong Y, Garver LS, Sim S, Dimopoulos G. 2010. Mosquito immune defenses against *Plasmodium* infection. *Developmental and Comparative Immunology* **34**:387–395 DOI 10.1016/j.dci.2009.12.005.

Clayton AM, Dong Y, Dimopoulos G. 2014. The *Anopheles* innate immune system in the defense against malaria infection. *Journal of Innate Immunity* **6**:169–181 DOI 10.1159/000353602.

Coatney GR, Collins WE, Warren M, Contacos PG. 1971. *The primate malarias*. Bethesda: US Department of Health, Education and Welfare.

Cohuet A, Osta MA, Morlais I, Awono-Ambene PH, Michel K, Simard F, Christophides GK, Fontenille D, Kafatos FC. 2006. *Anopheles* and *Plasmodium*: from laboratory models to natural systems in the field. *EMBO Reports* **7**:1285–1289 DOI 10.1038/sj.embor.7400831.

Crompton PD, Moebius J, Portugal S, Waisberg M, Hart G, Garver LS, Miller LH, Barillas-Mury C, Pierce SK. 2014. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annual Review of Immunology* **32**:157–187 DOI 10.1146/annurev-immunol-032713-120220.

De Almeida Oliveira G, Lieberman J, Barillas-Mury C. 2012. Epithelial nitration by a peroxidase/NOX5 system mediates mosquito antiplasmodial immunity. *Science* **335**:856–859 DOI 10.1126/science.1209678.

Derveaux S, Vandesompele J, Hellemans J. 2010. How to do successful gene expression analysis using real-time PCR. *Methods* **50**:227–230 DOI 10.1016/j.ymeth.2009.11.001.

Dzaki N, Ramli KN, Azlan A, Ishak IH, Azzam G. 2017. Evaluation of reference genes at different developmental stages for quantitative real-time PCR in *Aedes aegypti*. *Scientific Reports* **7**:43618 DOI 10.1038/srep43618.

Eldering M, Morlais I, van Gemert GJ, van de Vegte-Bolmer M, Graumans W, Siebelink-Stoter R, Vos M, Abate L, Roeffen W, Bousema T, Levashina EA, Sauerwein RW. 2016. Variation in susceptibility of African *Plasmodium falciparum*

malaria parasites to TEP1 mediated killing in *Anopheles gambiae* mosquitoes. *Scientific Reports* **6**:20440 DOI 10.1038/srep20440.

Fracisco S, Gettayacamin M, Tungtaeng A, Kozar M, O'Neil M, Craft C, Fernandes P. 2010. Anti-malarial activity of CEM-101, a fluoroketolide antimicrobial, in both blood stage and presumptive causal prophylactic mouse models. *Available at http:// www.cempra.com/ common/ pdf/ Posters/ ASTMH%20Nov%202010%20FRACISCO% 20CEM%20Poster_Final%2020101019.pdf* (accessed on 04 June 2017).

Fraiture M, Baxter RHG, Steinert S, Chelliah Y, Frolet C, Quispe-Tintaya W, Hoffmann JA, Blandin SA, Levashina EA. 2009. Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of *Plasmodium*. *Cell Host and Microbe* **5**:273–284 DOI 10.1016/j.chom.2009.01.005.

Frolet C, Thoma M, Blandin S, Hoffmann JA, Levashina EA. 2006. Boosting NF-κB-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. *Immunity* **25**:677–685 DOI 10.1016/j.immuni.2006.08.019.

Fu W, Xie W, Zhang Z, Wang S, Wu Q, Liu Y, Zhou X, Zhou X, Zhang Y. 2013. Exploring valid reference genes for quantitative real-time PCR analysis in *Plutella xylostella* (Lepidoptera: Plutellidae). *International Journal of Biological Sciences* **9**:792–802 DOI 10.7150/ijbs.5862.

Garver LS, Bahia AC, Das S, Souza-Neto JA, Shiao J, Dong Y, Dimopoulos G. 2012. *Anopheles* Imd pathway factors and effectors in infection intensity-dependent anti-*Plasmodium* action. *PLOS Pathogens* **8**:e1002737 DOI 10.1371/journal.ppat.1002737.

Garver LS, Dong Y, Dimopoulos G. 2009. Caspar controls resistance to *Plasmodium falciparum* in diverse anopheline species. *PLOS Pathogens* **5**:e1000335 DOI 10.1371/journal.ppat.1000335.

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. 2003. ExPASy: the proteomics server for in depth protein knowledge and analysis. *Nucleic Acids Research* **31**:3784–3788 DOI 10.1093/nar/gkg563.

Gupta L, Molina-Cruz A, Kumar S, Rodrigues J, Dixit R, Zamora RE, Barillas-Mury C. 2009. The STAT pathway mediates late-phase immunity against *Plasmodium* in the mosquito *Anopheles gambiae*. *Cell Host & Microbe* **5**:498–507 DOI 10.1016/j.chom.2009.04.003.

Habtewold T, Povelones M, Blagborough AM, Christophides GK. 2008. Transmission blocking immunity in the malaria non-vector mosquito *Anopheles quadriannulatus* species A. *PLOS Pathogens* **4**:e1000070 DOI 10.1371/journal.ppat.1000070.

Han YS, Thompson J, Kafatos FC, Barillas-Mury C. 2000. Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. *EMBO Journal* **19**:6030–6040 DOI 10.1093/emboj/19.22.6030.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology* **8**:Article R19 DOI 10.1186/gb-2007-8-2-r19.

Hillyer JF, Estevez-Lao TY. 2010. Nitric oxide is an essential component of the hemocyte-mediated mosquito immune response against bacteria. *Developmental and Comparative Immunology* **34**:141–149 DOI 10.1016/j.dci.2009.08.014.

Jaramillo-Gutierrez G, Rodrigues J, Ndikuyeze G, Povelones M, Molina-Cruz A, Barillas-Mury C. 2009. Mosquito immune responses and compatibility between *Plasmodium* parasites and anopheline mosquitoes. *BMC Microbiology* **9**:154 DOI 10.1186/1471-2180-9-154.

Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. 2008. NCBI BLAST: a better web interface. *Nucleic Acids Research* **36**:W5–W9 DOI 10.1093/nar/gkn201.

Khan MB, Liew JWK, Leong CS, Lau Y-L. 2016. Role of NF-kβ factor Rel2 during *Plasmodium falciparum* and bacterial infection in *Anopheles dirus*. *Parasites & Vectors* **9**:1–7 DOI 10.1186/s13071-015-1291-6.

Kozera B, Rapacz M. 2013. Reference genes in real-time PCR. *Journal of Appled Genetics* **54**:391–406 DOI 10.1007/s13353-013-0173-x.

Kumar S, Gupta L, Han YS, Barillas-Mury C. 2004. Inducible peroxidases mediate nitration of *Anopheles* midgut cells undergoing apoptosis in response to *Plasmodium* invasion. *Journal of Biological Chemistry* **279**:53475–53482 DOI 10.1074/jbc.M409905200.

Lapcharoen P, Komalamisra N, Rongsriyam Y, Wangsuphachart V, Dekumyoy P, Prachumsri J, Kajla MK, Paskewitz SM. 2012. Investigations on the role of a lysozyme from the malaria vector *Anopheles dirus* during malaria parasite development. *Developmental and Comparative Immunology* **36**:104–111 DOI 10.1016/j.dci.2011.06.010.

Le BV, Williams M, Logarajah S, Baxter RH. 2012. Molecular basis for genetic resistance of *Anopheles gambiae* to *Plasmodium*: structural analysis of TEP1 susceptible and resistant alleles. *PLOS Pathogens* **8**:e1002958 DOI 10.1371/journal.ppat.1002958.

Li MWM, Wang J, Zhao YO, Fikrig E. 2014. Innexin AGAP001476 is critical for mediating anti-*Plasmodium* responses in *Anopheles* mosquitoes. *Journal of Biological Chemistry* **289**:24885–24897 DOI 10.1074/jbc.M114.554519.

Luckhart S, Li K. 2001. Transcriptional complexity of the *Anopheles stephensi* nitric oxide synthase gene. *Insect Biochemistry and Molecular Biology* **31**:249–256 DOI 10.1016/S0965-1748(00)00144-2.

Luckhart S, Rosenberg R. 1999. Gene structure and polymorphism of an invertebrate nitric oxide synthase gene. *Gene* **232**:25–34 DOI 10.1016/S0378-1119(99)00121-3.

Luckhart S, Vodovotz Y, Cui L, Rosenberg R. 1998. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* **95**:5700–5705 DOI 10.1073/pnas.95.10.5700.

Manguin S, Garros C, Dusfour I, Harbach RE, Coosemans M. 2008. Bionomics, taxonomy, and distribution of the major malaria vector taxa of *Anopheles* subgenus Cellia in Southeast Asia: an updated review. *Infection, Genetics and Evolution* **8**:489–503 DOI 10.1016/j.meegid.2007.11.004.

Marchand RP, Culleton R, Maeno Y, Quang NT, Nakazawa S. 2011. Co-infections of *Plasmodium knowlesi*, *P*, falciparum, and *P. vivax* among humans and *Anopheles dirus* mosquitoes, Southern Vietnam. *Emerging Infectious Diseases* **17**:1232–1239 DOI 10.3201/eid1707.101551.

Mitri C, Jacques J-C, Thiery I, Riehle MM, Xu J, Bischoff E, Morlais I, Nsango SE, Vernick KD, Bourgouin C. 2009. Fine pathogen discrimination within the APL1 gene family protects *Anopheles gambiae* against human and rodent malaria species. *PLOS Pathogens* **5**:e1000576 DOI 10.1371/journal.ppat.1000576.

Mordecai EA, Paaijmans KP, Johnson LR, Balzer C, Ben-Horin T, De Moor E, McNally A, Pawar S, Ryan SJ, Smith TC, Lafferty KD. 2013. Optimal temperature for malaria transmission is dramatically lower than previously predicted. *Ecology Letters* **16**:22–30 DOI 10.1111/ele.12015.

Murdock CC, Blanford S, Luckhart S, Thomas MB. 2014. Ambient temperature and dietary supplementation interact to shape mosquito vector competence for malaria. *Journal of Insect Physiology* **67**:37–44 DOI 10.1016/j.jinsphys.2014.05.020.

Murdock CC, Moller-Jacobs LL, Thomas MB. 2013. Complex environmental drivers of immunity and resistance in malaria mosquitoes. *Proceedings of the Royal Society B: Biological Sciences* **280**:Article 20132030 DOI 10.1098/rspb.2013.2030.

Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF, Thomas MB. 2012. Complex effects of temperature on mosquito immune function. *Proceedings of the Royal Society B: Biological Sciences* **279**:3357–3366 DOI 10.1098/rspb.2012.0638.

Nakazawa S, Marchand RP, Quang NT, Culleton R, Manh ND, Maeno Y. 2009. *Anopheles dirus* co-infection with human and monkey malaria parasites in Vietnam. *International Journal of Parasitology* **39**:1533–1537 DOI 10.1016/j.ijpara.2009.08.005.

Neafsey DE, Waterhouse RM, Abai MR, Aganezov SS, Alekseyev MA, Allen JE, Amon J, Arca B, Arensburger P, Artemov G, Assour LA, Basseri H, Berlin A, Birren BW, Blandin SA, Brockman AI, Burkot TR, Burt A, Chan CS, Chauve C, Chiu JC, Christensen M, Costantini C, Davidson VL, Deligianni E, Dottorini T, Dritsou V, Gabriel SB, Guelbeogo WM, Hall AB, Han MV, Hlaing T, Hughes DS, Jenkins AM, Jiang X, Jungreis I, Kakani EG, Kamali M, Kemppainen P, Kennedy RC, Kirmitzoglou IK, Koekemoer LL, Laban N, Langridge N, Lawniczak MK, Lirakis M, Lobo NF, Lowy E, MacCallum RM, Mao C, Maslen G, Mbogo C, McCarthy J, Michel K, Mitchell SN, Moore W, Murphy KA, Naumenko AN, Nolan T, Novoa EM, O'Loughlin S, Oringanje C, Oshaghi MA, Pakpour N, Papathanos PA, Peery AN, Povelones M, Prakash A, Price DP, Rajaraman A, Reimer LJ, Rinker DC, Rokas A, Russell TL, Sagnon N, Sharakhova MV, Shea T, Simao FA, Simard F, Slotman MA, Somboon P, Stegniy V, Struchiner CJ, Thomas GW, Tojo M, Topalis P, Tubio JM, Unger MF, Vontas J, Walton C, Wilding CS, Willis JH, Wu YC, Yan G, Zdobnov EM, Zhou X, Catteruccia F, Christophides GK, Collins FH, Cornman RS, Crisanti A, Donnelly MJ, Emrich SJ, Fontaine MC, Gelbart W, Hahn MW, Hansen IA, Howell PI, Kafatos FC, Kellis M, Lawson D, Louis C, Luckhart S, Muskavitch MA, Ribeiro JM, Riehle MA, Sharakhov IV, Tu Z, Zwiebel LJ, Besansky

NJ. 2015. Mosquito genomics. Highly evolvable malaria vectors: the genomes of 16, *Anopheles* mosquitoes. *Science* **347**:Article 1258522 DOI 10.1126/science.1258522.

Omondi BA, Latorre-Estivalis JM, Rocha Oliveira IH, Ignell R, Lorenzo MG. 2015. Evaluation of reference genes for insect olfaction studies. *Parasites & Vectors* **8**:Article 243 DOI 10.1186/s13071-015-0862-x.

Omondi BA, Majeed S, Ignell R. 2015. Functional development of carbon dioxide detection in the maxillary palp of *Anopheles gambiae*. *Journal of Experimental Biology* **218**:2482–2488 DOI 10.1242/jeb.116798.

Paaijmans KP, Blanford S, Chan BH, Thomas MB. 2012. Warmer temperatures reduce the vectorial capacity of malaria mosquitoes. *Biology Letters* **8**:465–468 DOI 10.1098/rsbl.2011.1075.

Peterson TM, Luckhart S. 2006. A mosquito 2-Cys peroxiredoxin protects against nitrosative and oxidative stresses associated with malaria parasite infection. *Free Radical Biology and Medicine* **40**:1067–1082 DOI 10.1016/j.freeradbiomed.2005.10.059.

Pijpe J, Pul N, Van Duijn S, Brakefield PM, Zwaan BJ. 2011. Changed gene expression for candidate ageing genes in long-lived *Bicyclus anynana* butterflies. *Experimental Gerontology* **46**:426–434 DOI 10.1016/j.exger.2010.11.033.

Ponton F, Chapuis M-P, Pernice M, Sword GA, Simpson SJ. 2011. Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. *Journal of Insect Physiology* **57**:840–850 DOI 10.1016/j.jinsphys.2011.03.014.

Povelones M, Upton LM, Sala KA, Christophides GK. 2011. Structure-function analysis of the *Anopheles gambiae* LRIM1/APL1C complex and its interaction with complement C3-like protein TEP1. *PLOS Pathogens* **7**:e1002023 DOI 10.1371/journal.ppat.1002023.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* **7**:Article 539 DOI 10.1038/msb.2011.75.

Silver N, Best S, Jiang J, Thein SL. 2006. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Molecular Biology* **7**:33 DOI 10.1186/1471-2199-7-33.

Staley M, Dorman KS, Bartholomay LC, Fernandez-Salas I, Farfan-Ale JA, Lorono-Pino MA, Garcia-Rejon JE, Ibarra-Juarez L, Blitvich BJ. 2010. Universal primers for the amplification and sequence analysis of actin-1 from diverse mosquito species. *Journal of the American Mosquito Control Association* **26**:214–218 DOI 10.2987/09-5968.1.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**:Article Research0034 DOI 10.1186/gb-2002-3-7-research0034.

Vijay S, Rawat M, Adak T, Dixit R, Nanda N, Srivastava H, Sharma JK, Prasad GBKS, Sharma A. 2011. Parasite killing in malaria non-vector mosquito *Anopheles culicifacies* species B: implication of nitric oxide synthase upregulation. *PLOS ONE* **6**:e18400 DOI 10.1371/journal.pone.0018400.

Volohonsky G, Hopp AK, Saenger M, Soichot J, Scholze H, Boch J, Blandin SA, Marois E. 2017. Transgenic expression of the anti-parasitic factor TEP1 in the malaria mosquito *Anopheles gambiae*. *PLOS Pathogens* **13**:e1006113 DOI 10.1371/journal.ppat.1006113.

Vythilingam I, Sidavong B, Chan ST, Phonemixay T, Phetsouvanh R, Phompida S. 2005. Heavy natural oocyst infections in *Anopheles dirus* (Diptera: Culicidae) from Attapeu Province, Lao PDR. *International Journal of Tropical Insect Science* **25**:59–61 DOI 10.1079/IJT200554.

Wang M-H, Marinotti O, Zhong D, James AA, Walker E, Guda T, Kweka EJ, Githure J, Yan G. 2013a. Gene expression-based biomarkers for *Anopheles gambiae* age grading. *PLOS ONE* **8**:e69439 DOI 10.1371/journal.pone.0069439.

Wang Y, Wang Y, Zhang J, Xu W, Zhang J, Huang FS. 2013b. Ability of TEP1 in intestinal flora to modulate natural resistance of *Anopheles dirus*. *Experimental Parasitology* **134**:460–465 DOI 10.1016/j.exppara.2013.04.003.

White BJ, Lawniczak MKN, Cheng C, Coulibaly MB, Wilson MD, Sagnon NF, Costantini C, Simard F, Christophides GK, Besansky NJ. 2011. Adaptive divergence between incipient species of *Anopheles gambiae* increases resistance to *Plasmodium*. *Proceedings of the National Academy of Sciences of the United States of America* **108**:244–249 DOI 10.1073/pnas.1013648108.

Xu X, Dong Y, Abraham EG, Kocan A, Srinivasan P, Ghosh AK, Sinden RE, Ribeiro JMC, Jacobs-Lorena M, Kafatos FC, Dimopoulos G. 2005. Transcriptome analysis of *Anopheles stephensi-Plasmodium berghei* interactions. *Molecular and Biochemical Parasitology* **142**:76–87 DOI 10.1016/j.molbiopara.2005.02.013.

Zhang Z, Wang H, Hao C, Zhang W, Yang M, Chang Y, Li M. 2016. Identification, characterization and expression of methuselah-like genes in *Dastarcus helophoroides* (Coleoptera: Bothrideridae). *Gene* **7**:91 DOI 10.3390/genes7100091.