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Authors

Xavier, Nirmala
Marinotti, Osvaldo
James, Anthony A

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The accumulation of specific mRNAs following multiple blood meals in *Anopheles gambiae*

X. Nirmala*, O. Marinotti* and A. A. James*†

Departments of *Molecular Biology & Biochemistry, and †Microbiology & Molecular Genetics, University of California, Irvine, CA, USA

Abstract

One approach to genetic control of transmission of the parasites that cause human malaria is based on expressing effector genes in mosquitoes that disable the pathogens. Endogenous mosquito promoter and other *cis*-acting DNA sequences are needed to direct the optimal tissue-, stage- and sex-specific expression of the effector molecules. The mRNA accumulation profiles of eight different genes expressed specifically in the midgut, salivary glands or fat body tissues of the malaria vector, *Anopheles gambiae*, were characterized as a measure of their suitability to direct the expression of effector molecules designed to disable specific stages of the parasites. RT-PCR techniques were used to determine the abundance of the gene products and their duration following multiple blood meals. Transcription from the midgut-expressed carboxypeptidase-encoding gene, *AgCP*, follows a cyclical, blood-inducible expression pattern with maximum accumulation every 3 h post blood meal. Other midgut-expressed genes encoding a trypsin and chymotrypsin, *Antryp2* and *Anchym1*, respectively, and the fat body-expressed genes, *Vg1* and *Cathepsin*, also show a blood-inducible pattern of expression with maximum accumulation 24 h after every blood meal. Expression of the *Lipophorin* gene in the fat body and *apyrase* and *D7-related* genes (*AgApy* and *D7r2*) in the salivary glands is constitutive and not significantly affected by blood meals. Promoters of the midgut- and fat body-expressed genes may lead to maximum accumulation of antiparasite effector molecule transcripts after

multiple blood meals. The multiple feeding behaviour of *An. gambiae* thus can be an advantage to express high levels of antiparasite effector molecules to counteract the parasites throughout most of adult development.

Keywords: *Anopheles gambiae*, *Plasmodium*, multiple blood feeding, transmission blocking, antiparasite effector molecules, gene expression.

Introduction

Successful germline transformation of anophelines has increased the prospect of using genetically modified mosquitoes for blocking malaria transmission (Catteruccia *et al.*, 2000; Grossman *et al.*, 2001; Ito *et al.*, 2002). Antiparasite effector molecules such as monoclonal and single-chain antibodies, antiparasite toxins, molecules involved in immune responses and synthetic peptides are candidates for interfering with parasite development (Nirmala & James, 2003). These molecules target different stages of the parasites as they traverse the mosquito midgut, haemolymph and salivary glands. The efficiency of an effector molecule depends directly on its intrinsic toxicity and/or ability to block *Plasmodium* development, and also on its spatial and temporal expression pattern, which maximizes contact with the target stages of the parasite. Furthermore, continuous and abundant expression of antiparasite molecules is desirable in order to challenge the parasite for an extended period of time. Hence, considerable efforts have been invested to identify and characterize promoters of mosquito genes that are expressed in the primary tissues of vector–parasite interaction (James, 2002).

In *Anopheles gambiae* midguts, genes encoding products such as the peritrophic matrix protein (*Ag-Aper1*, Shen & Jacobs-Lorena, 1998) and carboxypeptidase (*AgCP*, Edwards *et al.*, 1997) are expressed at maximum levels early after a blood meal [3 h post blood meal (PBM)] whereas genes encoding *An. gambiae* trypsins (*Antryp1* and *Antryp2*, Muller *et al.*, 1993) and chymotrypsins (*Anchym1* and *Anchym2*, Vizioli *et al.*, 2001) are expressed at maximum levels much later (24 and 30 h PBM, respectively). DNA fragments containing the promoter elements of these genes are ideal to drive the expression of effector molecules targeting the early and later developmental stages of

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Received 11 August 2004; accepted after revision 1 October 2004. Correspondence: Dr Anthony A. James, Departments of Molecular Biology & Biochemistry and Microbiology & Molecular Genetics, 3205 McGaugh Hall, University of California, Irvine, CA 92697-3900, USA. Tel.: +1 949 824 5930; fax: +1 949 824 2814; e-mail: aajames@uci.edu

Figure 1. Experimental design for the multiple blood feeding of *Anopheles gambiae*.

Plasmodium in the lumen of the mosquito midgut. The *AgCP* gene promoter was used to drive the expression of the SM1 peptide (Ito *et al.*, 2002) and bee venom phospholipase A2 (Moreira *et al.*, 2002) in the midgut of transgenic *Anopheles stephensi*. In both cases the promoter efficiently expressed the transgenes and the effector molecules inhibited *Plasmodium berghei* oocyst formation.

The promoters of genes expressed in other mosquito tissues also can be used to drive the expression of antiparasite molecules. Fat-body-specific promoters derived from the *Vitellogenin* (*Vg*) genes of *Aedes aegypti* and *An. gambiae* can be used to synthesize and secrete effector molecules designed to counteract sporozoites as they migrate through the haemolymph from the oocysts to the salivary glands. The *An. gambiae* *Vg* promoter has yet to be used for the expression of exogenous genes in mosquitoes, but the *Ae. aegypti* *Vg* promoter directed efficiently the expression of defensin in the haemolymph of transgenic mosquitoes, conferring greater resistance to bacterial infection (Kokoza *et al.*, 2000). Other genes expressed in mosquito fat body, which are expressed in a constitutive (*Lipophorin*, van Heusden *et al.*, 1998) and blood-inducible pattern (*Cathepsin*, Cho *et al.*, 1999), have been cloned and characterized, providing alternative promoters for the expression of antiparasite molecules. None of these has been used yet to drive the expression of effector molecules in transgenic mosquitoes. Similarly, genes expressed in the mosquito salivary glands can provide promoters to control the expression of molecules with antiparasite activity. Several of these genes, such as *Maltase-like 1* (*Mal1*), *Apyrase* (*Apy* and *AgApy*) and *D7-related* (*D7r2*), as well as their promoters have been characterized in *Ae. aegypti* and *An. gambiae* (Arca *et al.*, 1999; Coates *et al.*, 1999; Lombardo *et al.*, 2000).

Most of the previous studies describing genes expressed in adult mosquitoes focused on variations in gene expression following a single blood meal. However, studies have shown that anophelines in the field may take multiple blood meals in a single gonotrophic cycle (Briegel & Horler, 1993; Koella *et al.*, 1998). In order to understand better the expression patterns of selected genes in adult *An. gambiae*

in conditions that resemble feeding behaviour in the field, we determined variations in mRNA accumulation of eight genes by quantitative RT-PCR after multiple blood-fed regimens.

Results

Multiple blood meals

Blood meals were given to three different cohorts of 200–400 *An. gambiae* females at intervals of 48 h (Fig. 1). The number of days elapsed before a blood-fed mosquito consumed a second blood meal was determined by transferring them to a new cage and providing access every 24 h to a blood meal (Fig. 2). Nearly 70% of the female mosquitoes took two blood meals within 24 h and 100% of them fed twice within a week (Fig. 3).

Figure 2. Experimental design to study the time taken for blood-fed *Anopheles gambiae* to ingest a second blood meal.

Figure 3. Number of days taken for blood-fed mosquitoes to take a second blood meal. Mosquitoes were fed using anaesthetized mice and blood-fed mosquitoes were removed to a separate cage. Blood meals were given to the fed mosquitoes every day until all the mosquitoes consumed a blood meal. A blood meal was not provided on day 5. Each time point in the data represents the average of three experiments defined by standard error bars.

Quantitative RT-PCR

The mRNA accumulation profiles were determined for *AgCP*, *Antryp2*, *Anchym1*, *Vg1*, *Cathepsin*, *Lipophorin*, *AgApy* and *D7r2* using RT-PCR. Reactions for each of the genes were performed using gene-specific primers and total mosquito RNA as template (yield equal to 5–13 µg per mosquito). Differences among individual mosquitoes in a population were determined by using RNA obtained from single mosquitoes. The gene products amplified by RT-PCR were analysed by gel electrophoresis and sequenced (data not shown). Their sizes and the absence of introns in the PCR products support the conclusion that the RNA samples were devoid of any contaminating genomic DNA.

Accumulation of mRNAs of genes expressed in the midgut

AgCP, *Antryp2* and *Anchym1* are transcribed in the midgut and showed expression profiles that were modulated by a blood meal (Fig. 4). The amount of *AgCP* transcript in mosquitoes increased significantly ($P < 0.01$), 2.5- to three-fold, 3 h PBM compared with levels detected in sugar-fed mosquitoes (Fig. 4A). The amount of mRNA dropped subsequently to basal levels at 24 h PBM and remained constant until at least 96 h PBM unless another blood meal was taken. Following a second blood meal, *AgCP* mRNA accumulation increased again to high levels 3 h PBM and dropped subsequently to sugar-fed levels. Thus, *AgCP* had a cyclical, inducible expression pattern if multiple blood meals were taken.

In contrast to *AgCP*, *Antryp2* and *Anchym1* had lower levels of mRNA accumulation in sugar-fed mosquitoes when normalized to *S17* mRNA abundance (Fig. 4B,C). *Antryp2* transcript levels in the midgut increased significantly

($P < 0.001$), five- to six-fold, 24 h PBM. The level of mRNA decreased at 48 h PBM and remained at the basal level until at least 96 h PBM. If a second blood meal was taken, *Antryp2* mRNA accumulated again to the maximum at 24 h PBM and dropped subsequently to sugar-fed levels. *Anchym1* gene expression was elevated sixty-three-fold ($P < 0.001$) 24 h PBM and subsequently decreased to basal levels. The results indicated that if mosquitoes were given multiple blood meals, *AgCP* transcript accumulation increased to a maximum 3 h PBM whereas that of *Antryp2* and *Anchym1* transcripts increased 24 h PBM resulting in continuous crests and troughs in their accumulation.

Accumulation of mRNAs from genes expressed in the fat body

The mRNAs of the fat body-expressed genes *Vg1* and *Cathepsin* accumulated significantly after each blood meal and this was interpreted to indicate the induction of novel transcription. Levels of *Vg1* mRNA increased 260-fold ($P < 0.01$) whereas those of *Cathepsin* were elevated thirty-five- to seventy-four-fold ($P < 0.01$) 24 h PBM, and subsequently dropped back to basal levels (Fig. 5A,B). Peak accumulation of mRNA was observed every 24 h PBM in adult mosquitoes taking multiple blood meals every 48 h. After each peak, mRNA levels decreased but increased again 24 h after the next blood meal. Accumulation of *Lipophorin* mRNA was not affected significantly by blood meals, with a 0.5-fold increase ($P < 0.2$) 24 h PBM and a subsequent decline to sugar-fed levels (Fig. 5C).

Accumulation of mRNAs of genes expressed in the salivary glands

Expression levels of the salivary gland genes *AgApy* and *D7r2* differed from each other and from the genes expressed in the midgut and fat body. *AgApy* mRNA accumulation remained almost unaltered after the first blood meal (Fig. 6A). This level increased 0.5- and one-fold 24 h after the second and third blood meals, respectively. However, if mosquitoes did not feed again, *AgApy* mRNA abundance dropped to levels even lower than that observed in sugar-fed females. In contrast, the levels of *D7r2* transcript decreased slightly after the first blood meal and continued to decline after subsequent blood meals (Fig. 6B). Overall, there was no statistically significant change in the levels of *AgApy* and *D7r2* accumulation.

Discussion

The development of transgenic mosquitoes expressing antiparasite effector molecules, and their ability to block or reduce malaria transmission under laboratory conditions, has fostered enthusiasm for releasing these insects as a tool to increase the efficiency of integrated malaria vector control programmes. Selection criteria for genes for which

Figure 4. Accumulation of AgCP (A), *Antryp2* (B) and *Anchym1* (C) transcripts in sugar-fed and multiple blood-fed *Anopheles gambiae* females normalized against the ribosomal protein gene transcript S17. The accumulation pattern was determined after ingestion of three consecutive blood meals at 48 h intervals. The normalized level of AgCP (A) is represented at 3 h PBM and every 24 h thereafter for 8 days after the first blood meal. Ratios of *Antryp2*/S17 (B) and *Anchym1*/S17 (C) are represented every 24 h PBM. Dotted lines represent the accumulation of each transcript after a single blood meal, recorded every 24 h for 4 days after the first blood meal. Each time point in the data represents the average expression of three individual mosquitoes defined by standard error bars. Absence of standard error bars indicates an average for two mosquitoes. Asterisks indicate $P < 0.01$ (A,C) and $P < 0.001$ (B).

promoters could drive sustainable expression of transgenes include those that have a high level of constitutive expression. However, such expression patterns and product accumulation may have a negative physiological effect on larval and pupal stages where effector molecule expression is not needed. Hence, the likely choices are blood meal-inducible genes that are specifically expressed in adult females (James *et al.*, 1999). In the experiments described here, we interpret differences in accumulation profiles to reflect changes in transcriptional activity of genes. In the absence of specific knowledge of mRNA stability in mosquitoes and

the relatively short time periods over which we observe a difference, we argue that this is a reasonable interpretation.

Genes expressed in the midgut

Midgut stages of *Plasmodium* have been the target of effector molecules in transgenic mosquitoes in an effort to curtail the parasite at the initial stages of infection. Promoters of genes from the carboxypeptidase, trypsin and chymotrypsin family whose products are involved in the proteolytic digestion of a blood meal are good candidates for this purpose. In *An. gambiae* midgut, AgCP is an 'early' gene

Figure 5. Accumulation of *Vg1* (A), *Cathepsin* (B) and *Lipophorin* (C) transcripts in sugar-fed and multiple blood-fed *Anopheles gambiae* females normalized against the ribosomal protein gene transcript *S17*. The accumulation pattern was determined after ingestion of three consecutive blood meals at 48 h intervals. The normalized level of each gene transcript is represented every 24 h for 8 days after the first blood meal. Dotted lines represent the accumulation of each transcript after a single blood meal, recorded every 24 h for 4 days after the first blood meal. Each time point in the data represents the average expression of three individual mosquitoes defined by standard error bars. Absence of standard error bars indicates an average of two mosquitoes. Asterisks indicate $P < 0.01$ (A,B) and $P < 0.2$ (C).

whose transcription product accumulates to maximum levels 3 h PBM (Edwards *et al.*, 1997). Expression of trypsin genes *Antryp1* and *Antryp2*, and chymotrypsin genes *Anchym1* and *Anchym2*, is inducible following a bloodmeal, and reaches a maximum accumulation between 24 and 30 h (Muller *et al.*, 1995; Vizioli *et al.*, 2001). An advantage of using an 'early' promoter in transgenesis is the expression of effector molecules in the midgut before the formation of peritrophic matrix (PM), which is formed around the blood bolus within 2 h of blood meal ingestion (Edwards *et al.*, 1997). As diffusion of macromolecules across the PM is speculated to be rate-limiting (Villalon *et al.*, 2003),

effector molecules expressed by *Antryp2* or *Anchym1* gene promoters may not gain access into the food bolus to interact with the ookinetes. In addition, these gene transcripts reach maximum accumulation 24 h PBM, whereas the ookinetes form 20 h PBM in the midgut lumen of *An. gambiae* (Ahmed *et al.*, 2001), providing a small window for the ookinetes to escape interaction with the effector molecule. However, because *An. gambiae* takes multiple blood meals at 24-h intervals (Klowden & Briegel, 1994), effector molecules driven by *Antryp2* and *Anchym1* may accumulate in abundance after first blood meal to interact with the parasites ingested during the second or third blood meal.

Figure 6. Accumulation of *AgApy* (A) and *D7r2* (B) transcripts in sugar-fed and multiple blood-fed *Anopheles gambiae* females normalized against the ribosomal protein gene transcript *S17*. The accumulation pattern was determined after ingestion of three consecutive blood meals at 48 h intervals. The normalized level of each gene transcript is represented every 24 h for 8 days after the first blood meal. Dotted lines represent the accumulation of each transcript after a single blood meal, recorded every 24 h for 4 days after the first blood meal. Each time point in the data represents the average expression of three individual mosquitoes defined by standard error bars. Absence of standard error bars indicates an average of two mosquitoes.

Genes expressed in the fat body

The promoters of blood meal-activated genes expressed in the fat body of mosquitoes are good candidates for directing the expression of effector molecules that target the sporozoite stage of *Plasmodium*. In *Ae. aegypti*, the fat body abundantly synthesizes proteins such as vitellogenin and cathepsin in the vitellogenic phase (Raikhel *et al.*, 2002). The *Vg* gene family in *An. gambiae* consists of four genes in genomes with the *Ikirara1* transposable element, and three in those without it (Romans *et al.*, 1995, 1998; Ahmed *et al.*, 2001). The transcript abundance of one of the vitellogenin genes (*Vg1*) increases to a maximum 24 h PBM as was confirmed in our study. In addition to *Vg1*, *Cathepsin* also is expressed in abundance in *An. gambiae*. Temporal control over the expression and accumulation of the mRNA of these genes is critical in targeting the sporozoites of *Plasmodium*. Because sporozoites reach the mosquito haemolymph between 8 and 16 days (depending on species and temperature) after an infective blood meal (Ghosh *et al.*, 2002), an effector molecule regulated by the inducible promoters of *Vg1* or *Cathepsin* should have a half-life of at least 1 to 2 weeks to encounter the parasite. Under multiple blood feeding conditions, expression of these genes

will remain high for a prolonged period to challenge the sporozoites that developed after the infective blood meal. In addition, it is known that the age of *An. gambiae* does not affect the number of probes or the probing time in laboratory conditions (Wekesa *et al.*, 1992), indicating that older mosquitoes harbouring sporozoites will continue to take blood meals and ensuring high levels of effector molecule expression.

Genes expressed in the salivary glands

Sporozoites invade the salivary glands where the infective forms of the parasite reside in the lumen for an extended period of time (Riehle *et al.*, 2003). The *AgApy* and *D7r2* genes are candidates to provide promoters to target salivary gland sporozoites because they are expressed strongly in the *An. gambiae* salivary glands (Ribeiro, 2003) and their transcript abundance is not significantly altered during blood meals. Because these genes are tissue- and stage-specific, and are also expressed constitutively in adult female mosquitoes (Arca *et al.*, 1999), they are ideal to drive the expression of effector molecules in the salivary glands.

Significance of multiple blood meals

Anautogenous mosquitoes such as *An. gambiae* require blood meals to initiate a gonotrophic cycle. The cycle starts

with the search for a host and is followed by blood ingestion and digestion, maturation of ovaries and oviposition (Klowden & Briegel, 1994). However, previous studies conducted with laboratory colonies and field-caught mosquitoes show that some anopheline species, including *An. gambiae*, take several blood meals during each gonotrophic cycle (Boreham & Garrett-Jones, 1973; Burkot *et al.*, 1988; Klowden & Briegel, 1994). Those mosquitoes taking a second blood meal could correspond to a small proportion of the population that was not successful in complete engorgement during the first feeding bout, and therefore needed to acquire more nutrients via another blood meal to complete oogenesis. Our observations show that most mosquitoes will seek blood meals every 24 h, even when fully engorged after a first blood meal. Because laboratory- and field-based studies are consistent with a multiple feeding behaviour, sustainable expression of effector molecules can be achieved by using promoters of blood meal-induced or up-regulated genes. In addition, the likelihood of a *Plasmodium*-infected mosquito taking multiple blood meals is higher than that in uninfected mosquito (Koella *et al.*, 1998), indicating that multiple blood-feeding behaviour can be an advantage to drive the expression of effector molecules.

Conclusion

The availability of genetic resources and technology for modifying mosquitoes has advanced the transmission blocking efforts to focus on the introduction of genetically modified mosquitoes in the field. Challenges facing the release of transgenic mosquitoes include fitness costs of refractoriness, possible resistance to antiparasite genes and development of a drive mechanism to sustain the transgene in the population (Riehle *et al.*, 2003). Sustainable expression of antiparasite effector molecules is one such challenge that can be overcome by choosing appropriate promoters for transgenesis.

In transgenic mosquitoes generated so far, no single effector molecule has inhibited *Plasmodium* transmission completely (Nirmala & James, 2003). Therefore, it is likely that more than one kind of effector molecule driven by different promoters should be used for effective transmission blocking. An ideal vector-incompetent mosquito will target parasite ligands in all stages of *Plasmodium* development in the midgut, haemolymph and salivary glands. Ookinetes that escape effector molecules in the midgut can be targeted as sporozoites by genes expressed in the fat body and salivary glands. Blood-meal-inducible genes *AgCP*, *Antryp2* and *Anchym1* in the midgut, *Vg1* and *Cathepsin* in the fat body are ideal candidates for use in transgenic mosquitoes because of their tissue- and stage-specific expression pattern. *AgApy* and *D7r2* are not affected by blood meal ingestion but are strong promoters

that could be used to drive effector molecule expression in the salivary glands.

Experimental procedures

Mosquitoes

The *Pink-eye* strain of *An. gambiae* (Githeko *et al.*, 1992) was maintained at 25 °C, 75–85% relative humidity and 18/6-h light–dark cycles. Larvae were fed on finely powdered fish food (Tetramin) mixed 1 : 1 with yeast powder. Adults were fed on raisins and water *ad libitum*. Adult female mosquitoes were fed using anaesthetized mice.

The first blood meal was provided to 3- to 4-day-old sugar-fed mosquitoes and those that had fed to repletion were separated from the cohort and transferred to a new cage. A second blood meal was given after 48 h and the females that fed a second time were transferred to a new cage. Similarly, a third blood meal was provided 48 h later and the blood-fed females were separated. After each blood-meal, 20–25 blood-fed females were removed to a new cage to analyse gene expression after each meal. From these groups, three to four females were collected at 24, 48, 72 and 96 h for analysis of gene expression by one-step RT-PCR. Care was taken to create a laboratory model to mimic natural conditions for mosquitoes. During the entire procedure, a cup filled with water was placed in all the cages to facilitate oviposition and males were added to each cage of blood-fed females.

Determination of mRNA abundance by RT-PCR

mRNA abundance was quantified according to Justice *et al.* (2003). In brief, total RNA was extracted from whole bodies of female mosquitoes using Trizol (Gibco/BRL). Oligonucleotide primers for each gene of interest were designed from the *An. gambiae* database (Table 1). Wherever possible, primer pairs were derived from two different exons with an intervening intron in order to detect amplification products from genomic DNA that may have precipitated during the RNA preparation. Primers were designed to yield amplification products ranging in size between 500 bp and 1.0 kb. The gene encoding the ribosomal protein, *S17*, was used as a standard in the amplification reactions, and its primer pair produced a 250 bp fragment from its RNA.

Cycle-controlled one-step RT-PCR reactions were performed using the one-step RT-PCR kit (Qiagen) in 50 µl volumes containing 100–200 ng of total RNA. Each amplification reaction contained 20 pmol of each of the gene-specific and *S17* primers. Reactions were performed in an MJ Research thermal cycler as follows: reverse transcription at 50 °C for 30 min, activation of *Taq* polymerase at 95 °C for 15 min, followed by thirty-six cycles of denaturation at 94 °C for 30 s, annealing at 66 °C for 30 s and extension at 72 °C for 1 min. In order to quantify the amplified products in a phosphorimager, 1 µCi each of $\alpha^{32}\text{P}$ -dATP and $\alpha^{32}\text{P}$ -dCTP (3000 Ci/mm, Amersham Pharmacia) were added to each 50 µl RT-PCR reaction. Ten-microlitre aliquots were removed from each of the reactions during the annealing phase at cycles 24, 28, 32 and 36 and mixed with the gel-loading buffer containing sucrose and bromophenol blue. Ten-microlitre samples were loaded on 3% agarose gels, which then were dried after electrophoresis on to Zeta-probe GT Genomic Tested Blotting Membranes (Bio-Rad) and exposed on Kodak BioMax film. For quantification of the signals, dried gels were exposed in phosphorimager cassettes and scanned in a phosphorimager (Molecular Dynamics). Quantification

Table 1. Genes, oligonucleotide primers, and specifics of gene sequences

Gene	Primer name	Primer sequence (5'–3')	NCBI accession no. ENSANGT no.	bp amplified
<i>Antryp2</i>	T2 For T2 Rev	CCTCCTGCTGGCTGT TCTGGTTGCGG GGTGAT T TCTCCGAACCACATGTAGGC	Z18890 00000027737	615
<i>Anchym1</i>	C1 For C1 Rev	CTCCTGGTAGTAAGTGCAGCCAAGGTGCC CCCAATCATGATAGTACGAGACACGGGC	Z18887 00000023046	715
<i>AgCP</i>	CBP For CBP Rev	ATGCGAAGGTGCGTGCACTGGCCG GTAGCGCTAAGCCACCAACCCGC	AF000953 00000021052	716
<i>Vg1</i>	Vg1 For Vg1 Rev	GGTGACCGACTTGT TGCTCATGTCC CTACCATCCATCT TCGCGCTGTGTCG	AF281078 00000012892	1077
<i>Cathepsin</i>	Cat For Cat Rev	GGGATGT TGC GACTCGAGCTACGCCG CCCCACTGTGCACCCAGGAAT TGGC	Not available 00000012222	585
<i>Lipophorin</i>	LIP For LIP Rev	CGCTAGTGCTGATTCAAAGTGTGTCCG GGGTCACGAAGCTGT TCGAGAGCG	Not available 00000018348	504
<i>AgApy</i>	Apy For Apy Rev	TGGCACTGCGCAT TCT TGCACCG GTGAGCAATGCGAATGGGCG	AJ237705 00000015382	970
<i>D7r2</i>	D72 For D72 Rev	CTGAGCGTTGGACTGGTCTGGTG GAT T TCC T T GATCAGCGCTGCC	AY045760 00000018371	624
<i>S17</i>	S17 For S17 R2	GGGTCGTGTACGTACCAAGACG CATAGT TATCGCGACGCTCACG	AF164153 00000013205	250

was performed with ImageQuant software (Molecular Dynamics) and tabulated in Microsoft Excel. Only values of signal intensities that were increasing linearly in proportion to the amplification cycle were used for further analysis. Signal intensities that reached a saturation point during amplification were not used. The ratio of signal intensities between the product of each gene and S17 was calculated for individual mosquitoes and values are represented as average of three mosquitoes. Standard error and *t*-tests were performed in Microsoft Excel.

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