Published in final edited form as:
Comp Biochem Physiol B Biochem Mol Biol. 2008 August ; 150(4): 403–408.

Silencing the genes for dopa decarboxylase or dopachrome conversion enzyme reduces melanization of foreign targets in *Anopheles gambiae*

Susan M. Paskewitz* and **Olga Andreev**
Department of Entomology, University of Wisconsin, Madison, Wisconsin, USA

Abstract

The production of melanin is a complex biochemical process in which several enzymes may play a role. Although phenoloxidase and serine proteases are clearly key components, the activity of other enzymes, including dopa decarboxylase and dopachrome conversion enzyme may also be required. We tested the effect of knockdown of gene expression for these two enzymes on melanization of abiotic targets in the mosquito, *Anopheles gambiae*. Knockdown of dopa decarboxylase and dopachrome conversion enzyme resulted in a significant reduction of melanization of Sephadex beads at 24h after injection. Knockdown of a third enzyme, phenylalanine hydroxylase, which is involved in endogenous production of tyrosine, had no effect on bead melanization. Quantitative analysis of gene expression demonstrated significant upregulation of phenylalanine hydroxylase, but not the other two genes, following injection.

Keywords

Anopheles gambiae; insect immunity; melanization; mosquito

Introduction

The pigment melanin is widespread in the animal kingdom, with varied functional roles ranging from formation of surface color patterns and protection against ultraviolet radiation to internal defense against pathogens. The latter role is apparent in many insects where melanized capsules have been observed around a wide variety of foreign objects including abiotic targets, bacteria, nematode worms, parasitoids, and protists (Salt 1963; 1970). In mosquitoes, melanization functions as a natural mechanism of resistance against human malaria parasites in *Anopheles culicifacies* (Adak et al., 2006) and against the nematode *Brugia malayi* in *Armigeres subalbatus* (Infanger et al., 2004). Melanization of malaria parasites has also been artificially selected in a laboratory colony of *An. gambiae* (Collins et al., 1986; Paskewitz et al., 1988) and a significant body of work has developed to identify the molecular determinants of this phenotype (Blandin et al., 2004; Volz et al., 2006; Kumar et al., 2003).

*Corresponding author: Dr. Susan M. Paskewitz, 237 Russell Labs, 1630 Linden Dr. Department of Entomology, Madison, Wi, 53706, USA. Tel (608) 262-1269; Fax (608) 262-3322, E-mail: paskewit@entomology.wisc.edu

The biochemical pathways leading to melanin formation are complex and can involve multiple branches (True, 2003; Christensen et al., 2005; Nappi and Christensen 2005). Central to melanin biosynthesis in insects are the phenoloxidase enzymes (PO). Tyrosinase-type POs exist in zymogen forms (ProPO) which can be activated by serine proteases (termed proPO activating protease (PAP) or proPO activating enzyme (PPAE)). Other serine proteases are involved in activating PPAE and in initiating the activation cascade (Ji et al., 2004). Once PO is activated, a series of enzyme-catalyzed and spontaneous reactions may occur. As a first step, the amino acid tyrosine must be hydroxylated to produce dopa by the action of PO or tyrosine hydroxylase. Tyrosine can be dietary or can be derived endogenously by the action of phenylalanine hydroxylase (PAH) on its substrate, phenylalanine. Once dopa is formed, it can then be oxidized to dopaquinone. PO also catalyzes this reaction. Dopaquinone spontaneously converts to dopachrome, which can be decarboxylated by dopachrome conversion enzyme (DCE; also known as dopachrome isomerase) to form 5,6-dihyroxyindole which is then oxidized by PO (Christensen et al., 2005). The resulting compound will polymerize to form melanin. Dopamine, derived from dopa via the action of dopa decarboxylase (DDC), can also serve as the catecholamine substrate for melanin production. Identifying the enzymes and accessory proteins that contribute to melanin formation during different physiological processes remains an active area of research (Huang et al., 2005; Jiang et al., 2005; Volz et al., 2006; Tang et al., 2006; Warr et al., 2006).

The molecular basis of melanogenesis in mosquitoes has been studied through gene silencing experiments which have provided clear demonstrations of the functional roles of some of the enzymes noted above. In culicine mosquitoes, knockdown of *PO I* (Shiao et al., 2001), *Ddc* (Huang et al., 2005a), *Dce* (Huang et al., 2005b), or *Pah* (Infanger et al., 2004) reduces melanization of nematode worms. However, the extent to which these observations hold true for different targets and for anopheline mosquitoes has not been determined. Genetic studies have demonstrated that melanization of different targets are often controlled by different pathways even within a species (Zheng et al., 2003; Tang et al., 2006). Melanization in *Anopheles gambiae* can be easily induced in response to the injection of an abiotic target, Sephadex beads (Paskewitz and Riehle, 1994). Thus, in this study, we used the bead system and RNAi-mediated gene silencing to investigate the role of three enzymes, DDC, DCE and PAH, in the melanization response.

Materials and Methods

Mosquitoes

The G3 strain of *A. gambiae* was used for all experiments. The G3 strain originated from mosquitoes collected from The Gambia. The G3 strain has the ability to melanize malaria parasites and was used in genetic selection of a line that fully melanizes a wide range of *Plasmodium* species and strains (Collins et al. 1986). Mosquitoes were reared as described previously (Paskewitz et al., 1999).

Identification of A. gambiae DDC, PAH, and DCE genes

Sequences for all three genes were identified by database screening. Accession numbers are: dopa decarboxylase **AF063021**, dopachrome conversion enzyme **AJ459959**, and phenylalanine hydroxylase **AF283273** (Oduol et al., 2000).

RNA interference

An *in vitro* transcription template was produced using a two-step PCR protocol (Dudley et al., 2002). First, a pair of 35 bp primers, each designed to include 15 bp of T7 promoter sequence (in bold) plus 20 bp of the target gene sequence, were used to amplify a product from mosquito cDNA. The gene-specific primers were: **Dopa decarboxylase** 5′

CGACTCACTATAGGGACACCGGGCCAGCCTTCGAG3′ and 5′ **CGACTCACTATAGGG**CTGCTGCTGCTGGCGTTCAT; **Phenylalanine hydroxylase** 5′ **CGACTCACTATAGGG**GTGCGTCTTCTGCGGTGTGA3′ and 5′ **CGACTCACTATAGGG**GAAGCTGACCAACCCGATCA3′; **Dopachrome conversion enzyme** 5′**CGACTCACTATAGGG**CTGGGACAGGATTGCGTAAA3′ and 5′ **CGACTCACTATAGGG**TCGACAGGCCCGCGTGCTTC3′.

Because injection of dsRNA can suppress the mosquito immune response (Blandin et al., 2002), we used an exogenous gene for control injections. A cloned gene for green fluorescent protein (GFP) was used to produce control dsRNA as described above. Primers included 15 bp of T7 promoter sequence plus 20 bp GFP sequence were used to amplify a product using the phMFGP vector (Promega, Madison, WI, USA) as the template DNA. The gene specific primers were (5′**CGACTCACTATAGGG**CGTGATCAAGCCCGACA-3′ and 5′-**CGACTCACTATAGGGTGGG**CTTCGGCGTGCT-3′).

Amplification parameters were an initial denaturation step of 92°C for 1 min followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, 72°C for 45 s. After a final 10 min extension at 72°C, reactions were held at.4°C until they were frozen. Each PCR product was purified using a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA). Purified products then were used in a second PCR reaction with a primer containing a full T7 site: 5′ TAATACGACTCACTATAGGG3′. The resulting product was again purified using the Qiagen Gel Extraction kit and 1-2 μg of the product were used as template for transcription. The Megascript RNAi kit (Ambion, Austin, TX, USA) was used for transcription and the production of dsRNA following the manufacturer's directions. All dsRNA preparations were quantified by measuring absorbance at 260 nm, checked for integrity on an agarose gel, and stored at -20°C.

For most experiments, mosquitoes (0-36 h after eclosion) were injected with 0.1 μg dsRNA (in 0.2 μL dH_2O) using pulled glass needles. To increase the efficiency of knockdown, some cohorts were injected with up to 1.2 μg of dsRNA.

Four days after dsRNA for GFP or for DDC, DCE and PAH were injected, we injected one CM Sephadex bead into each mosquito as described in the next section. After an incubation period of 24 h, we collected the carcasses for analysis of the efficacy of knockdown and also removed beads for scoring melanization. Each experiment was independently replicated three or more times and at least 75 mosquitoes each were assessed for control and knockdown treatments.

Sephadex bead inoculation

Four days after dsRNA injections, mosquitoes were injected with CM-25 Sephadex beads (40-120 μm; Sigma-Aldrich, St. Louis, MO, USA) as previously described (Chun et al., 1995; Gorman et al., 1998). Briefly, beads were hydrated in mosquito saline prior to inoculation. Each bead was aspirated with less than 0.5 μL of saline into a pulled glass needle and then injected into the hemocoel of a female that had been anesthetized on ice. One bead was injected per mosquito at the junction between the thoracic and abdomen using a mouth aspirator. For all experiments, mosquitoes were placed into small humidified plastic cages supplied with 10% sucrose and allowed to recover in an incubator at 70-80% relative humidity and 25-26°C

Semiquantitative RT-PCR

Total RNA from carcasses of at least 10 female mosquitoes was extracted and used to verify that the knockdown was successful. Samples were extracted with the AquaPure RNA isolation

kit (BioRad, Hercules, CA,, USA) and then treated with amplification grade DNaseI (Invitrogen, San Jose, CA, USA). First strand cDNA was synthesized using oligo dT and reverse transcriptase Superscript III (Invitrogen) and used for subsequent PCR reactions.

Samples were subjected to semi-quantitative RT-PCR after first using the ribosomal S7 gene (5′-TGCTGCAAACTTCGGCTAT-3′ and 5′-CGCTATGGTGTTCGGTTCC-3′) to normalize the samples (30 sec at 92°C, 56°C, and 1.0 min at 72°C for 21 cycles). To amplify enzymes genes, gene-specific primers (10 pmol) were used in 20 μL reactions with Amplitaq (1 unit, Promega, Madison, WI, USA), 200 μM dNTP, 2.0 mM $MgCl_2$. Conditions for the PCR reactions were: 30 s at 92°C, 56°C, and 1.0 min at 72°C for 35 cycles for CLIPB9. The same conditions and 30 cycles were used for all others.

Primers used for each gene were: **DDC** 5′-GTGCGTCTTCTGCGGTGTGA-3′ and 5′-GAAGCTGACCAACCCGATCA-3′; **PAH** 5′-CAAACAGTGCTGGCGCTAGA-3′ and 5′-TGCGGCCCGAAGCTAACCAC-3′; **DCE** 5′-CATCCGGTCCGAGATTGTCC-3′ and 5′-TGTGCGACATCCCGAACGAG-3′.

Real-Time PCR

Whole mosquitoes were used for RNA extraction following bead injection. Total RNA was isolated as described above and was treated with RQ1 DNAse (Promega) to remove contaminating genomic DNA. RNA concentration was measured with micro-spectrophotometry (Nanodrop NT1000, Thermofisher, Waltham, MA, USA). Next, removal of DNA from the RNA samples was confirmed by real-time PCR using housekeeping gene primer sets (no cDNA control). Samples that yielded threshold cycle (C_t) values larger than 33 were deemed acceptable (Rotenberg et al. 2006). One hundred ng of high-quality total RNA was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The resulting cDNA was diluted 1:5 and stored at 4°C. In our experience, freeze/thaw cycles result in degraded cDNA samples.

Primers for internal reference genes (ribosomal protein *S7*, *RPS3*, *RPS31*, tropomyosin, and actin) and the three target genes (*Ddc*, *Dce*, and *Pah*) were designed using the Beacon designer software (Premier Biosoft International, Palo Alto, CA, USA). The program setting "avoid template structure" was chosen to limit primer sequences to regions of little secondary template structure. Primers chosen were: **actin-forward** CAGTCCAAGCGTGGTATC **actin-reverse** GTTAGCCTTCGGGTTCAG; **RPS7-forward** CCTATGGTGTTCGGTTCC, **RPS7-reverse** GATCGCCTTCTTGTTGTTG; **Tropomyosin-forward** GAACGGATTCAGCAGGTG, **Tropomyosin-reverse** TTCTCAGCATCTTCAAGCC; **RPS31-forward** GACAGCATTTGGGACGATTC, **RPS31-reverse** GTTGATTAGGACAGACTTTACCG; **RPS3-forward** CTATGAAGGTTATGCTCTGC, **RPS3-reverse** CATCAGGTAGTCGGTCAG; **DDC-forward** GTGGGAAGAGGTGATGGC, **DDC-reverse** GTAGGCGTGGAACTTGGG; **DCE-forward** GCGGTCCACCATAAGAAC, **DCE-reverse** AGTTAGGATAGGGCTTCAGG; **PAH-forward** CGACTACAAGGATAACGAAGC, **PAH-reverse** GTACGACAGGATCTGGTTGG.

The primers were synthesized by IDT Technologies (Coralville, IA, USA). The performance of these primers (PCR efficiency and standard curves) was tested on five dilutions of cDNA prepared as described above. All standard curves were generated from triplicate reactions of the five-fold dilution series of cDNA. Suitable internal reference gene primer sets were chosen on the basis of i) primer efficiencies that were close to 100% and ii) threshold cycles were within 5 cycles of those seen for the target genes. Both reference and target primers exhibited

similar efficiencies as determined using a dilution series of cDNA derived from *A. gambiae* total RNA (uninoculated adult female control).

Real-time PCR was carried out using an iCycler machine (BioRad) and analyzed using the iQ software package (BioRad). All reactions were performed in triplicate 25 μL volumes using iQ SYBRGreen Supermix (BioRad). A master mix was prepared for each primer set containing SYBRGreen and an appropriate volume of each primer to yield a final primer concentration of 200 nM. The reaction conditions were enzyme activation and well factor determination at 95°C for 3 min followed by 40 cycles of 95°C for 10 s (denaturation) and 58°C for 45 s (annealing and elongation); the melt curve protocol began immediately after amplification and consisted of 95°C for 1 min, followed by 55°C for 1 min and then 80-10s steps of 0.5°C increases at each step. Threshold values for threshold cycle (Ct) determination were generated automatically by the iCycler software. The absence of primer artifacts was determined from the melt curve profile of the PCR products.

The stability of the reference genes in comparison to each other during the treatments was analyzed using the BestKeeper program (Pfaffl et al., 2004; MS-Excel based Q-PCR data analysis program).

Results

Effect of bead injection on transcript levels

We examined whether transcript abundance for the three enzymes changed during activation of the melanization pathway. Three independent cDNA samples were prepared from mosquitoes that were harvested following injection with one CM Sephadex bead. Each experiment included four time points (preinjection (0h), and 3h, 6h and 24h after bead injection).

Because large variation in housekeeping gene expression occurs in vertebrate systems (Vandesompele et al., 2002; Brunner et al., 2004; Radonic et al., 2004), we tested five genes for stability following bead injection. For this initial analysis, we used total RNA input as the reference (Sindelka et al., 2006). Actin exhibited large increases in threshold levels when comparing between time 0 and 24h, suggesting that gene expression may be suppressed during the response to wounding and injection. The other four genes exhibited a minimal increase of <2 fold change in expression at all times. Although gene expression was stable, *RPS3* and *S7* transcripts were much more abundant than the target genes (a difference greater than 5 cycles) which is undesirable for quantitative PCR. Tropomyosin and *RPS31* were expressed at levels similar to the target genes but tropomyosin did not produce consistent results at the 24 h timepoint. Thus, *RPS31* was chosen as the best reference gene. Interestingly, three of the stable housekeeping genes do not vary much in terms of functionality; they are ribosomal proteins involved in protein biosynthesis. Similar results have recently been reported in an evidence-based approach to housekeeping gene identification for vertebrates (De Jong et al., 2007)

Figure 1 provides the results of all three trials for each target gene because the relative fold change in expression varied somewhat between experiments (interassay variation). This was apparent especially at the 24 h timepoint, which demonstrates the risk of relying on RNA from a single experiment for Q-PCR. Earlier timepoints were more consistent. Compared with the preinoculation controls (0 h), *Pah* was upregulated at 3 and 6 h postinoculation by an average of 3 fold. *Dce* was upregulated by approximately 2.5 fold at 3 and 6 h. *Ddc* transcript levels were not significantly altered during these trials (1.8 fold increase or less). These experiments do not discriminate between changes due to the injection alone (wounding) or to introduction of the bead. However, melanin formation is induced in either case.

Gene silencing

The RT-PCR results demonstrate that significant knockdown of all three enzymes occurred (Fig. 2, 3, 4). Knockdown of *Dce* resulted in reduced melanization of CM Sephadex beads (Fig. 2). Over three experiments, the average proportion of mosquitoes that completely melanized beads was 28% compared with 64% when the control dsGFP was injected (Student's t-test, $p<0.05$). While we found unmelanized beads in only 6% of the *GFP* controls, 28% of the *Dce* knockdowns failed to initiate melanization on the bead surface. In other experiments, the amount of dsRNA for *Dce* was increased from 0.1 to 0.66 μg, resulting in further reduction in the expression of this gene but this did not significantly alter the bead melanization outcomes.

Silencing of *Ddc* also resulted in significantly reduced melanization of CM Sephadex beads (Figure 3). When we used mosquitoes for dsRNA injection two days after eclosion, the average proportion of mosquitoes that completely melanized beads was 38% in *Ddc* knockdowns compared with 73% for *GFP* controls (Student's t-test, $p<0.05$). Two additional experiments were carried out using mosquitoes that were injected with dsRNA on the first day after eclosion but here there were no differences between *Ddc* knockdowns and controls. RT-PCR indicated that knockdown of *Ddc* was successful in all replicates, suggesting that newly eclosed mosquitoes may have additional resources for bead melanization that render the activity of *Ddc* redundant.

Silencing of *Pah* did not reduce melanization of CM-Sephadex beads in comparison with *GFP* controls (Fig. 4).

Discussion

Melanization can play a key role in containing and killing foreign organisms in mosquitoes. Inactivation of this immune mechanism can result in enhanced survival of malaria parasites (Volz et al., 2006) and filarial worms (Shiao et al., 2001). Melanization can also be elicited by bacteria (Hillyer et al., 2003a, b) and abiotic particles like Sephadex beads (Paskewitz and Riehle, 1994). In *Anopheles gambiae*, there is clear evidence that several components of the melanization pathway are essential for melanization of both abiotic targets and *Plasmodium berghei* (Blandin et al., 2004; Paskewitz et al., 2006; Volz et al., 2006; Warr et al., 2006). These studies identified specific serine proteases and a recognition protein as critical upstream regulators of the process. Silencing of serine proteases *CLIPB4* and *CLIPB8* (Paskewitz et al., 2006; Volz et al. 2006) and *TEP1* (Warr et al., 2006) strongly reduce bead and parasite melanization. Other proteins of unknown function (leucine rich repeat immune protein) also regulate the bead and parasite melanization response (Warr et al., 2006; Osta et al., 2004).

Pah, which functions to produce tyrosine from phenylalanine, could be an additional upstream regulator of melanization if stores of this essential substrate are limited. Some evidence supports a role for this enzyme in mosquito immunity. *Pah* transcript abundance increases following immune challenge with the dog heartworm, *Dirofilaria immitis* or bacteria in *Aedes aegypti* (Johnson et al., 2003) and following lipopolysaccharide injection of *An. gambiae* (Oduol et al., 2000). We also found convincing evidence of upregulation of this gene following bead injection. Knockdown of *Pah* in *Ar. subalbatus* or *Ae. aegypti* was reported to reduce melanization of injected *D. immitis*, although a dsRNA control was not performed (Infanger et al., 2004). Knockdown of *Pah* had no effect on bead melanization in *An. gambiae*. However, examination of bead melanization at times earlier than 24 h might reveal a delay in the process. It is also possible that the PAH protein in *An. gambiae* was not significantly reduced in spite of strong reduction of the transcript. In *Ar. subalbatus* subjected to RNAi, the PAH protein appeared unusually stable and did not decrease until after injection of *D. immitis* (Infanger et al., 2004). Finally, it is possible that the silencing of PAH had no impact on bead melanization

but would have a significant impact on melanization of another challenge type. Further investigation will be needed to resolve this issue in *An. gambiae*.

Enzymes that function downstream of PO have also been investigated in mosquitoes. Biochemical analyses demonstrated the presence of dopachrome conversion enzyme in *Ae. aegypti* hemolymph and its role in accelerating melanization *in vitro* (Li et al., 1994). Johnson and colleagues (2001) observed upregulation of transcripts following inoculation of *D. immitis* in *Ae. aegypti*. Silencing of *Dce* in *Ar. subalbatus* by RNAi resulted in reduction of melanization of *D. immitis* (Huang et al., 2005b). In *An. gambiae, Dce* transcripts were slightly upregulated following infection by the human malaria parasite, *Plasmodium falciparum* (Dong et al., 2006) and following bead injection (this study). A significant reduction of bead melanization occurred after knockdown of *Dce*. This effect is small relative to that seen for *TEP1* and serine proteases. In accordance with the biochemical data, we conclude that *Dce* accelerates but is not an essential factor for melanization of these abiotic targets in *An. gambiae*.

For dopa decarboxylase, silencing was accomplished through engineering of Sindbis virus and introduction of antisense virus into *Ar. subalbatus* (Huang et al., 2005a). Silencing resulted in an initial reduction of melanization of *D. immitis* microfilariae that had been injected into the hemocoel. However, longer incubation times revealed a progressive increase in melanization. *Ddc*-silenced *Armigeres* mosquitoes also exhibited high mortality, abnormal movement and overfeeding, conditions that are attributable to the role of DDC in producing dopamine, a neurotransmitter. We monitored for these effects but, surprisingly, did not observe them for *An. gambiae*. Quantitative PCR demonstrated a significant rise in the relative expression of *Ddc* in comparison with actin in *Ar. subalbatus* mosquitoes 48 h after blood feeding or injection of *D. immitis* (Huang et al., 2005a). *Ddc* transcripts increased in *An. gambiae* following exposure to oxidative stress (Dimopoulos et al., 2002) but not following bead injection (this study). Silencing of *Ddc* resulted in a small but significant decrease in the percentage of beads that were fully melanized by 24 h.

Our findings, together with those of previous studies, suggest that melanization pathways in mosquito immunity usually use DDC and DCE to accelerate formation of the end products. Since melanization clearly limits the ability of some mosquito species to transmit pathogens (Adak et al., 2006; Christensen et al. 2005; Infanger et al., 2004), further investigation of this complex phenomenon may provide avenues for development of novel control strategies for these parasites.

Acknowledgements

We thank Professor Kyle Willis, University of Wisconsin, Department of Plant Pathology, for his help in establishing and interpreting quantitative PCR protocols. We thank Jun Wang for sharing her expertise in this area as well. Finally, we acknowledge the work of Beth Schadd, who reared all mosquitoes used in these experiments. This work was supported in part by a grant from the National Institutes of Health to SMP (AI46031).

References

Adak T, Singh OP, Nanda N, Sharma VP, Subbarao SK. Isolation of a Plasmodium vivax refractory Anopheles culicifacies strain from India. Trop. Med. Int Health 2006;11:197–203. [PubMed: 16451344]

Blandin S, Moita LF, Kocher T, Wilm M, Kafatos FC, Levashina EA. Reverse genetics in the mosquito Anopheles gambiae: targeted disruption of the Defensin gene. EMBO Reports 2002;3:852–856. [PubMed: 12189180]

Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, Kafatos FC, Levashina EA. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. Cell 2004;116:661–670. [PubMed: 15006349]

Brunner AM, Yakovlev IA, Strauss SH. Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol 2004;4:1–7. [PubMed: 15005814]

Christensen BM, Li J, Chen CC, Nappi AJ. Melanization immune responses in mosquito vectors. Trends Parasitol 2005;21:192–199. [PubMed: 15780842]

Chun J, Riehle M, Paskewitz SM. Effect of mosquito age and reproductive status on melanization of Sephadex beads in Plasmodium-refractory and susceptible strains of Anopheles gambiae. J. Invert. Pathol 1995;66:11–17.

Collins FH, Sakai RK, Vernick KD, Paskewitz SM, Seeley DC, Miller L, Collins WE, Campbell CC, Gwadz RW. Genetic selection of a Plasmodium-refractory strain of the malaria vector Anopheles gambiae. Science 1986;234:607–610. [PubMed: 3532325]

De Jonge HJM, Fehrmann RSN, de Bont ESJM, Hofstra RMW, Gerbens F, Kamps WA, de Vries EGE, van der Zee AGJ, Meerman GJ, Elst AT. Evidence-based selection of housekeeping genes. PLoS ONE 2007;2:898–910.

Dimopoulos G, Christophides GK, Meister S, Schultz J, White KP, Barillas-Mury C, Kafatos FC. Genome expression analysis of Anopheles gambiae: Responses to injury, bacterial challenge and malaria infection. Proc. Natl. Acad. Sci. USA 2002;99:8814–8819. [PubMed: 12077297]

Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, Dimopoulos G. Anopheles gambiae immune responses to human and rodent Plasmodium parasite species. PLoS Pathogens 2006;6:1–13.

Dudley NR, Labbe JC, Goldstein B. Using RNA interference to identify genes required for RNA interference. Proc. Natl. Acad. Sci. USA 2002;99:4191–4196. [PubMed: 11904378]

Gorman MJ, Schwartz AM, Paskewitz SM. The role of surface characteristics in eliciting humoral encapsulation of foreign bodies in Plasmodium-refractory and susceptible strains of Anopheles gambiae. J. Insect Physiol 1998;44:947–954. [PubMed: 12770431]

Hillyer JF, Schmidt SL, Christensen BM. Rapid phagocytosis and melanization of bacteria and Plasmodium sporozoites by hemocytes of the mosquito Aedes aegypti. J. Parasitol 2003a;89:62–69. [PubMed: 12659304]

Hillyer JF, Schmidt SL, Christensen BM. Hemocyte-mediated phagocytosis and melanization in the mosquito Armigeres subalbatus following immune challenge by bacteria. Cell Tissue Res 2003b; 313:117–127. [PubMed: 12838409]

Huang CY, Chou SY, Bartholomay LC, Christensen BM, Chen CC. The use of gene silencing to study the role of dopa decarboxylase in mosquito melanization reactions. Insect Mol. Biol 2005a;14:237–244. [PubMed: 15926892]

Huang CY, Christensen BM, Chen CC. Role of dopachrome conversion enzyme in the melanization of filarial worms in mosquitoes. Insect Mol. Biol 2005b;14:675–682. [PubMed: 16313567]

Infanger LC, Rocheleau TA, Bartholomay LC, Johnson JK, Fuchs J, Higgs S, Chen CC, Christensen BM. The role of phenylalanine hydroxylase in melanotic encapsulation of filarial worms in two species of mosquitoes. Insect Biochem. Mol. Biol 2004;34:1329–1338. [PubMed: 15544946]

Ji C, Wang Y, Guo X, Hartson S, Jiang H. A pattern recognition serine proteinase triggers the prophenoloxidase activation cascade in the tobacco hornworm, Manduca sexta. J. Biol. Chem 2004;279:34101–34106. [PubMed: 15190055]

Jiang H, Wang Y, Gu Y, Guo X, Zou Z, Scholz F, Trenczek TE, Kanost MR. Molecular identification of a bevy of serine proteases in Manduca sexta hemolymph. Insect Biochem Mol Biol 2005;35:931–943. [PubMed: 15944088]

Johnson JK, Li J, Christensen BM. Cloning and characterization of a dopachrome conversion enzyme from the yellow fever mosquito, Aedes aegypti. Insect Biochem. Mol. Biol 2001;31:1125–1135. [PubMed: 11520691]

Johnson JK, Rocheleau TA, Hillyer JF, Chen CC, Li J, Christensen BM. A potential role for phenylalanine hydroxylase in mosquito immune responses. Insect Biochem. Mol. Biol 2003;33:345–354. [PubMed: 12609519]

Kumar S, Christophides GK, Cantera R, Charles B, Han YS, Meister S, Dimopoulos G, Kafatos FC, Barillas-Mury C. The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae. PNAS 2003;100:14139–14144. [PubMed: 14623973]

Li J, Zhao X, Christensen BM. Dopachrome conversion activity in Aedes aegypti: significance during melanotic encapsulation of parasites and cuticular tanning. Insect Biochem. Mol. Biol 1994;24:1043–1049. [PubMed: 7703986]

Nappi AJ, Christensen BM. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. Insect Biochem. Mol. Biol 2005;35:443–459. [PubMed: 15804578]

Oduol F, Xu J, Niare O, Natarajan R, Vernick KD. Genes identified by an expression screen of the vector mosquito Anopheles gambiae display differential molecular immune response to malaria parasites and bacteria. Proc. Natl. Acad. Sci. USA 2000;97:11397–11402. [PubMed: 11005829]

Osta MA, Christophides GK, Kafatos FC. Effects of mosquito genes on Plasmodium development. Science 2004;303:2030–2032. [PubMed: 15044804]

Paskewitz SM, Riehle MA. Response of Plasmodium-refractory and susceptible strains of Anopheles gambiae to inoculated Sephadex beads. Develop. Comp. Immunol 1994;18:369–375.

Paskewitz SM, Brown MR, Lea AO, Collins FH. Ultrastructure of the encapsulation of Plasmodium cynomolgi (B strain) on the midgut of a refractory strain of Anopheles gambiae. J. Parasitol 1988;74:432–439. [PubMed: 3379524]

Paskewitz SM, Reese-Stardy S, Gorman MJ. An easter-like serine protease from Anopheles gambiae exhibits changes in transcript levels following immune challenge. Insect Mol. Biol 1999;8:329–338. [PubMed: 10469250]

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol. Lett 2004;26:509–515. [PubMed: 15127793]

Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem. Biophys. Res. Commun 2004;313:856–862. [PubMed: 14706621]

Rotenberg D, Thompson TS, German TL, Willis DK. Methods for effective real time RT-PCR analysis of virus-induced gene silencing. J. Virol Meths 2006;138:49–59.

Salt G. The defence reactions of insects to metazoan parasites. Parasitol 1963;53:527–642.

Salt G. The cellular defence reactions of insects. Cambridge Monogr Exp Biol 1970;16:1–118.

Shiao SH, Higgs S, Adelman Z, Christensen BM, Liu SH, Chen CC. Effect of prophenoloxidase expression knockout on the melanization of microfilariae in the mosquito Armigeres subalbatus. Insect Mol. Biol 2001;10:315–321. [PubMed: 11520354]

Sindelka R, Ferjentsik Z, Jonak J. Developmental expression profiles of Xenopus laevis reference genes. Dev Dyn 2006;235:754–758. [PubMed: 16397894]

Tang H, Kambris Z, Lemaitre B, Hashimoto C. Two proteases defining a melanization cascade in the immune system of Drosophila. J. Biol. Chem 2006;281:28097–28104. [PubMed: 16861233]

True JR. Insect melanism: the molecules matter. Trends Ecol. Evol 2003;18:640–647.

Vandesompele K, De Preter F, Pattyn B, Poppe N, Van Roy A, De Paepe F, Speleman. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;34:1–11.

Volz J, Muller HM, Zdanowicz A, Kafatos FC, Osta MA. A genetic module regulates the melanization response of Anopheles to Plasmodium. Cell. Microbiol 2006;8:1392–1405. [PubMed: 16922859]

Warr E, Lambrechts L, Koella JC, Bourgouin C, Dimopoulos G. Anopheles gambiae immune responses to Sephadex beads: involvement of anti-Plasmodium factors in regulating melanization. Insect Biochem. Mol. Biol 2006;36:769–778. [PubMed: 17027843]

Zheng L, Wang S, Romans P, Zhao H, Luna C, Benedict MQ. Quantitative trait loci in Anopheles gambiae controlling the encapsulation response against Plasmodium cynomolgi Ceylon. BMC Genetics 2003;4:16–26. [PubMed: 14577840]

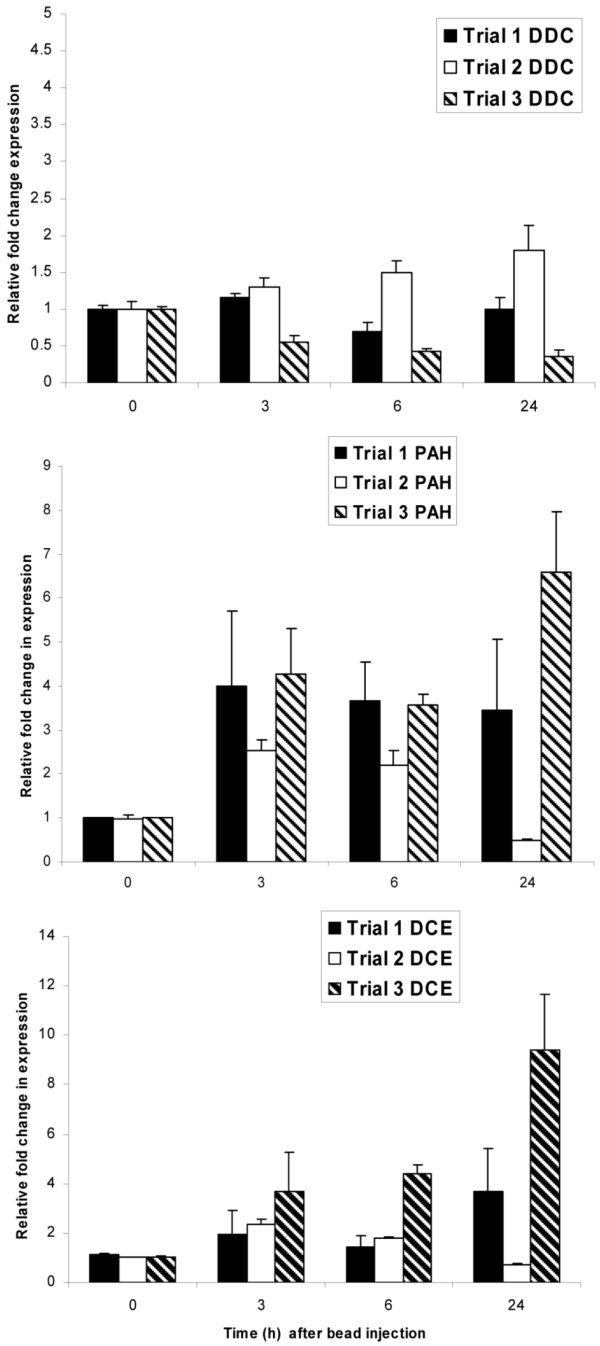

Figure 1.

Real time PCR analysis of expression profiles for three genes, dopa decarboxylase (DDC), dopachrome conversion enzyme (DCE) and phenylalanine hydroxylase (PAH) following bead injection. Results of three replicate assays are provided for each gene. Ribosomal protein S31 was used to normalize the samples. Expression at times 3, 6 and 24 h was determined relative to time 0 (arbitrarily set at 1); time 0 results are included to illustrate variation between replicate experiments. Note that the relative expression scale differs for each gene.

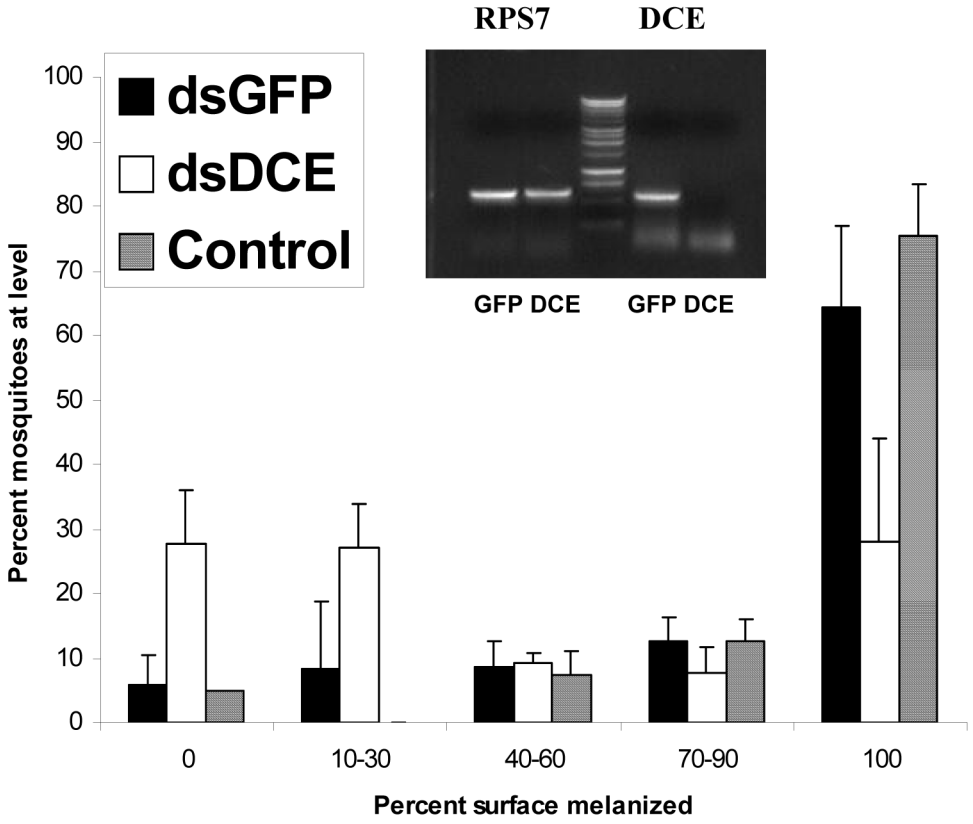

Figure 2.

Effect of RNAi mediated silencing of dopa decarboxylase on melanization of CM-Sephadex beads in *Anopheles gambiae*. A frequency distribution for 5 categories of increasing strength of melanization is presented. Control mosquitoes were not injected with double stranded RNA. GFP mosquitoes were injected with dsGFP. Inset: ethidium bromide stained gel demonstrating the degree of knockdown as measured by RT-PCR. RPS7 is used as a calibration control, resulting in approximately equal transcript levels of this nontarget gene in both dsDDC and dsGFP treatments.

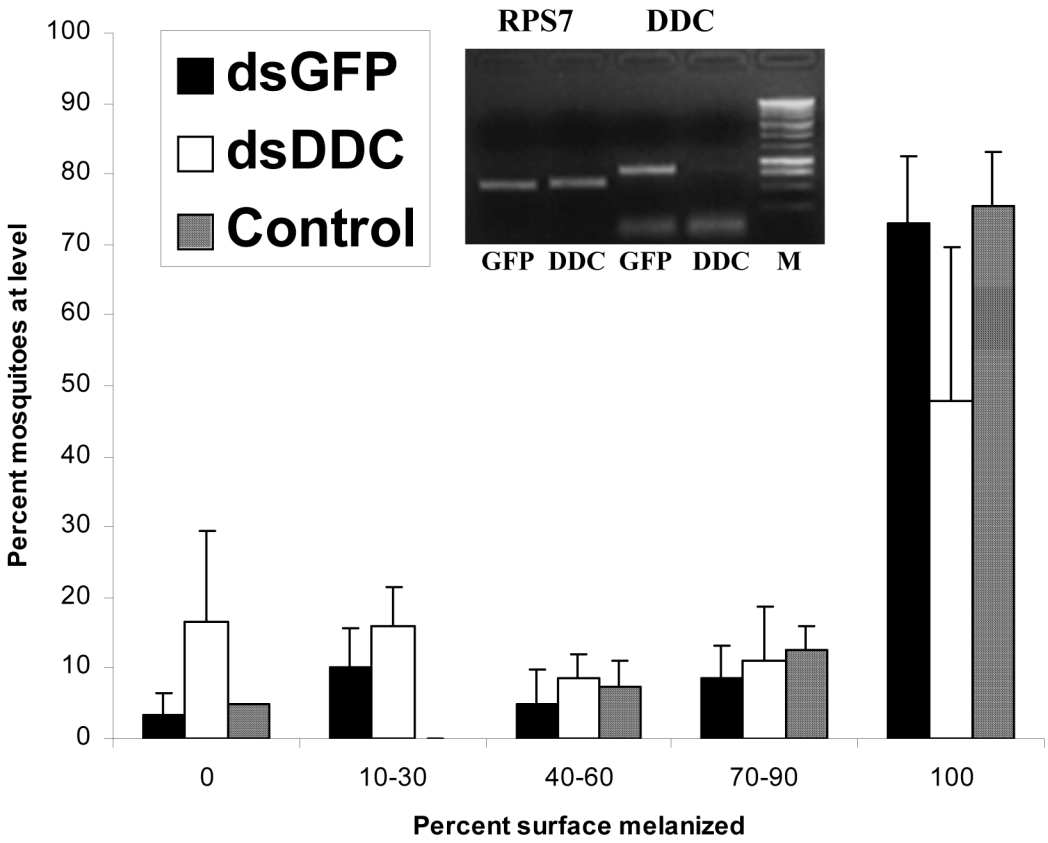

Figure 3.
Effect of RNAi-mediated silencing of dopachrome conversion enzyme on melanization of CM-Sephadex beads in *Anopheles gambiae*. A frequency distribution for 5 categories of increasing strength of melanization is presented. Control mosquitoes were not injected with double stranded RNA. GFP mosquitoes were injected with dsGFP. Inset: ethidium bromide stained gel demonstrating the degree of knockdown as measured by RT-PCR. RPS7 is used as a calibration control, resulting in approximately equal transcript levels of this nontarget gene in both dsDCE and dsGFP treatments.

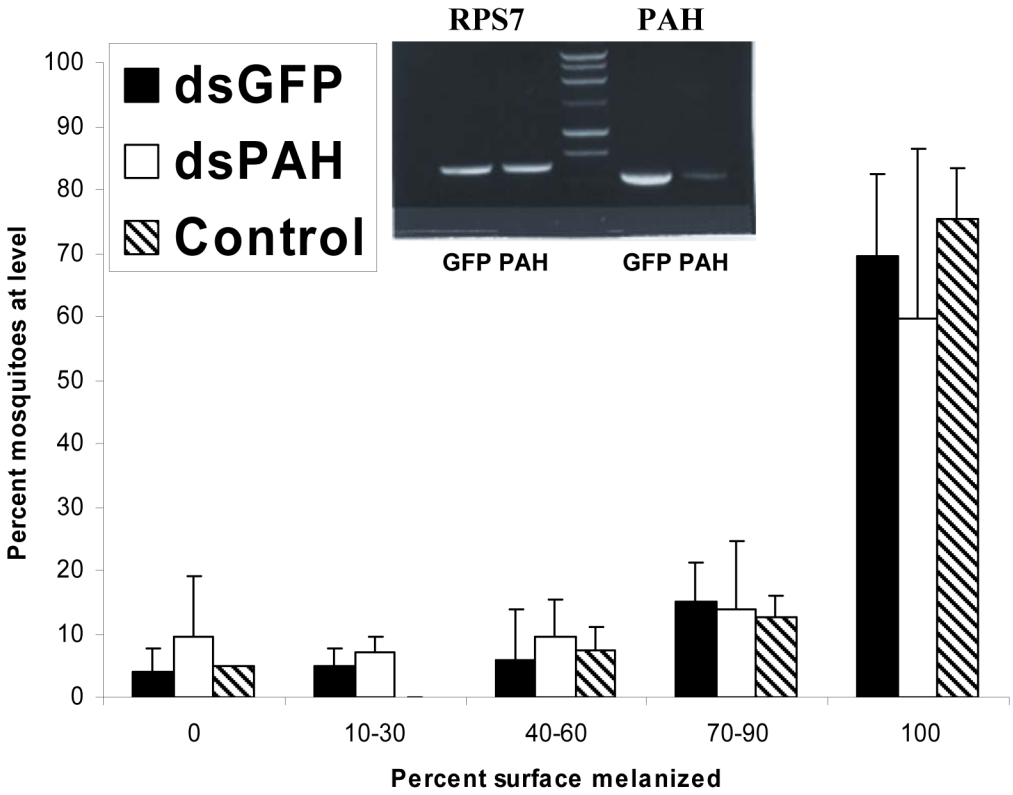

Figure 4.

Effect of RNAi-mediated silencing of phenylalanine hydroxylase on melanization of CM-Sephadex beads in *Anopheles gambiae*. A frequency distribution for 5 categories of increasing strength of melanization is presented. Control mosquitoes were not injected with double stranded RNA. GFP mosquitoes were injected with dsGFP. Inset: ethidium bromide stained gel demonstrating the degree of knockdown as measured by semiquantitative RT-PCR. RPS7 is used as a calibration control, resulting in approximately equal transcript levels of this nontarget gene in both dsPAH and dsGFP treatments.