Cytochrome *c* Gene and Protein Expression: Developmental Regulation, Environmental Response, and Pesticide Sensitivity in *Aedes aegypti*

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ABSTRACT Cytochrome *c* is a highly conserved protein that is found in many multicellular and unicellular organisms. Cytochrome *c* is a critical intermediate in apoptosis: a controlled form of cell death that kills cells as part of their natural process of development and in response to environmental condition. To detect whether cytochrome *c* of the mosquito *Aedes aegypti* (L.) (*AeaCytC*) is developmentally regulated, we used quantitative real-time polymerase chain reaction (PCR) to examine *AeaCyt*C gene expression levels in different developmental stages of *Ae. aegypti*. Quantitative real-time PCR showed that *AeaCytC* was expressed in each developmental stage, at different points in time, and it was highly expressed in teneral female *Ae. aegypti*. *Ae. aegypti* cytochrome *c* protein (AeaCYTC) was detected only in adult mosquitoes, not in early developmental stages of *Ae. aegypti*. We also investigated the effect of certain environmental factors (e.g., temperature, UV-light, and permethrin insecticide) on *AeaCytC* gene and AeaCYTC protein expression in adult mosquitoes, and we found that response varied with age. These results suggest that *AeaCytC* gene and AeaCYTC protein play functional roles in the development of *Ae. aegypti* and the differential expression of cytochrome *c* has potential as a biomarker for environmental and chemical stress.

KEY WORDS cytochrome *c*, *Aedes aegypti*, development, environment, permethrin

Cytochrome *c* is a small highly conserved heme protein that has a key role in mitochondrial electron transfer and onset of apoptosis in most organisms (Bernardi and Azzone 1981, Cai and Jones 1998, Zhang et al. 1998, Crofts et al. 1999, Murgida and Hildebrandt 2004, Nakagawa et al. 2007). Cytochrome *c* is crucial for mitochondrial regulation of apoptosis and functions by mechanisms that have been conserved through evolution. The role of cytochrome *c* in signaling cell death emphasizes the importance for defining the biochemical pathways for its interactions in diverse biological systems. In the mosquito *Aedes aegypti* (L.), a primary vector of dengue and yellow fever viruses, the role of cytochrome *c* during development is unknown.

Cytochrome *c* is not only at the very center of life in the oxygen-rich world but also a crucial factor in programmed cell death (Goodsell 2004). Many lethal agents target the mitochondria, and they cause release of cytochrome *c* and other proapoptotic proteins into the cytoplasm (Orrenius et al. 2007). Cytochrome *c* release is initiated by dissociation of the hemoprotein from the inner mitochondrial membrane.

As in all organisms, development of mosquitoes requires multigene regulation (Severson et al. 2004, Fontenille et al. 2005, Raibaud et al. 2006, Strode et al. 2006, van den Hurk et al. 2007), and they are affected by environmental conditions, such as temperature and radiation, as well as challenges from parasites (Harizanova et al. 2005, Dong et al. 2006). The current study examines the role of cytochrome *c* (*AeaCytC* gene and AeaCYTC protein) in developmental regulation, environmental response, and pesticide sensitivity in *Ae. aegypti*, for which the genome sequence has been mapped (Nene et al. 2007).

Materials and Methods

Mosquito Strains. *Aedes aegypti* (Orlando, FL strain, maintained since 1952) were reared in the insectary of the Mosquito and Fly Research Unit at the USDA–ARS Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), Gainesville, FL. Female mosquitoes were used for all experiments using methods described previously (Pridgeon et al. 2007). Females were not blood fed, but they were given a sucrose source during the course of the experiments.

RNA Extraction. All developmental stages of *Ae. aegypti* (i.e., eggs, larvae, pupae, and adults) were collected at numerous time points within each stage. Total RNAs were extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Poly(A)$^+$ RNA was isolated with Oligotex-dT (QIAGEN, Valencia, CA). RNA samples

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were quantified by SmartSpec Plus spectrophotometry (Bio-Rad, Hercules, CA).

UV-Light Experiments. *Ae. aegypti* adult females, aged 3, 10, and 17 d, were exposed to a germicidal lamp (30-W, G30TB, General Electric) at the light intensity 1,000 $\mu W/cm^2$ for the time course study. Sixty individuals (30 for RNA and 30 for protein extraction) were collected at 0, 15, 30, 90, and 180 min after UV-light treatment.

Heat-Shock Experiments. *Ae. aegypti* adult females, aged 2 and 9 d, were exposed to three temperatures (24, 37, and 42°C) and 56% ± 1.5% RH in an environmental chamber (L-C Incubator, Lab-Line Instruments, Inc., Melrose Park, IL) for the time course study. Sixty individuals (30 for RNA and 30 for protein extraction) were collected at 0, 15, 30, and 180 min after heat stress.

Permethrin Experiments. *Ae. aegypti* adult females, aged 3 and 10 d, were treated topically with permethrin at 1.25×10^{-5} μg per mosquito (LD_{10}) as described by Pridgeon et al., 2007. Sixty females (30 for RNA and 30 for protein extraction) were collected at 0, 1, 3, 6, and 12 h after permethrin treatment. An untreated control group was exposed to acetone only (Pridgeon et al. 2007).

cDNA Synthesis. A 5-μg aliquot of purified RNA was reverse transcribed in a 20-μl reaction volume by using cloned avian myeloblastosis virus first-strand cDNA synthesis system for real-time polymerase chain reaction (PCR) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The reaction was terminated by heat inactivation at 95°C for 5 min. The reactions were diluted adding 80 μl of double-distilled H_2O. The cDNA samples for each developmental stage and different experiments were stored at −20°C.

Design of Gene-Specific Primers for Real-Time PCR. To design gene-specific primers, a detailed analysis of the nucleotide sequence of the *AaeCytC* gene (National Center for Biotechnology Information [NCBI] accession no. DQ440105.1 or GI: 94468577) was performed using PRIMER3-Design Primer Pairs and Probes program from Biology Workbench (http://workbench.sdsc.edu). The primers for *Ae. aegypti* actin gene (NCBI accession no. DQ440059) were also designed for an internal control and comparison.

Real-Time PCR Amplification. The quantitative real-time PCR assay for cytochrome *c* gene expression in *Ae. aegypti* (designated *AeaCytC*) was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) in a volume of 15 μl on a 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR mixture consisted of 1 μl diluted cDNA, 0.5 μM primers, and 1× master mix. In every real-time reverse transcription (RT)-PCR run, *ACTIN* was used as an internal control to normalize for variation in the amount of cDNA template. The PCR primers used were AEA-CYTC-12 F (5′-AGCTGGAGACGTTGAGAAGG-3′) and AEA-CYTC-254R (5′-ATCTTCGTGCCAGGGATGTA-3′). The PCR primers for *ACTIN* are Actin-152 F (5′-AGGACTCGTACGTCGGTGAC-3′) and Actin-590R (5′-CGTTCAGTCAGGATCTTC-3′). The PCR thermal cycling parameters were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. This was followed by the dissociation stage at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. This experiment was replicated three times. Relative expression levels were calculated as follows: First, *AeaCytC* transcript levels relative to a standard (*ACTIN*) by using the formula $\Delta C_T = C_T$ (*AeaCytC*) $- C_T$ (*ACTIN*). Second, an average ΔC_T value for each sample was calculated. Third, the sample with lowest relative expression (highest ΔC_T value), was used as the standard for the comparison of expression levels. Then relative expression levels were calculated using the equation $100 \times 2^{-[\text{average } \Delta CT]}$ (Portereiko et al. 2006). The data were further analyzed in Excel (Microsoft, Redmond, WA). To determine a significant difference between the data, SigmaStat software (Systat Software, Inc., Point Richmond, CA) was used for comparing two groups of data.

Immunoblotting of Protein Extracts. Total proteins were isolated from early eggs, late eggs, first and fourth instars, early and late pupae, and early and late adult females. The samples (30 mosquitoes each) collected from heat stress, UV-light, and permethrin treatments were divided into two groups: one for RT-PCR RNA extractions and the other for protein extraction. The cell lysis buffer contained 50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, and 1% IGEPAL, and protease inhibitors cocktail. Total proteins were isolated by adding 0.5 ml of cell lysis buffer to 15 mosquitoes (≈50 mg), which were homogenized with a pestle. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (BCA protein assay kit) according to the manufacturer's protocol (Pierce Chemical, Rockford, IL). Protein concentrations were quantified using Multiskan MCC (Thermo Electron Corporation Fisher, Vantaa, Finland).

Samples for polyacrylamide gel electrophoresis were loaded with equal protein content (25 μg) on precast NuPAGE 12% Bis-Tris gels and using NuPAGE MES SDS running buffer (Invitrogen). Proteins separated electrophoretically were transferred to nitrocellulose membrane using an Xcell blotting apparatus (Invitrogen). *Ae. aegypti* cytochrome *c* protein (designated AeaCYTC proteins were detected using mouse monoclonal (7H8.2C12) to cytochrome *c* (ab28137) (Abcam Inc., Cambridge, MA) antibody. SuperSignal West Pico Chemiluminescent Substrate, an enhanced chemiluminescent substrate for detection of horseradish peroxidase was used for detecting the signal. The nitrocellulose membrane was exposed to the film. The nitrocellulose membrane was stripped using Restore Western blot stripping buffer (Pierce Chemical). The nitrocellulose membrane was reprobed with rabbit polyclonal to Actin (ab1801) antibody (Abcam Inc.). The images were scanned by an Epson Perfection 4490 Photo scanner (digital ICE technology) and further processed by Adobe Photoshop CS2 (Adobe Systems, Mountain View, CA). The scanned images were converted into digital data using the UN-SCAN-IT (Silk Scientific Inc., Orem, UT).

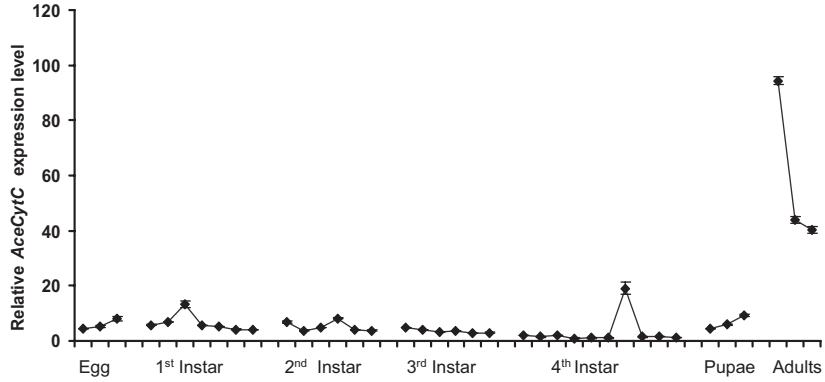

Fig. 1. *AeaCytC* gene expression levels in eggs, larvae, pupae, and adult females by using quantitative real-time PCR. The ages of eggs are 1, 3, and 6 d, respectively. First instar: 7, 10, 23, 30, 33, 36, and 39 h posthatch; second instar: 48, 51, 54, 57, 60, and 63 h posthatch; third instar: 72, 75, 78, 81, 84, and 87 h posthatch; fourth instar: 96, 99, 102, 105, 108, 111, 129, 132, 145, and 148 h posthatch; pupae: 154, 157, and 169 h posthatch; adult: 1-d-old female (i.e., 12 d posthatch); 7-d-old female (i.e., 19 d posthatch); and 14-d-old female (i.e., 26-d-posthatch).

Results

AeaCytC Gene Regulation in Different Developmental Stages of *Ae. aegypti*. To understand how cytochrome *c* is regulated during the development of *Ae. aegypti*, we examined *AeaCytC* relative expression levels in eggs, larvae, pupae, and adult females using quantitative real-time PCR (Fig. 1). During the development of *Ae. aegypti*, the real-time PCR data showed that the lowest *AeaCytC* RNA expression was in the fourth-stage larvae (105 h posthatch). We set this point as standard = 1, and the *AeaCytC* RNA expression in other stages was compared with this point. *AeaCytC* RNA expression in *Ae. aegypti* eggs was relatively low compared with that in the adults. In mid-late first-stage larvae (23 h posthatch), *AeaCytC* expression was slightly higher (≈13.3 ± 1.6 times >105 h posthatch) than in the other specimens of early (7 and 10 h posthatch) and late (36 and 39 h posthatch) of the first-stage larvae. In the mid-late fourth larvae (129 h posthatch), *AeaCytC* expression was 19-fold higher (19 ± 2.03) than in the specimens of larval stages 1 (except mid-late), 2, and 3 examined. However, the RNA relative expression level of *AeaCytC* increased dramatically in teneral adult mosquitoes, and it was 94.37 ± 1.34 times more than the lowest point in fourth-stage larvae. Expression of *AeaCytC* in teneral adults (1-d-old female, i.e., 12 d posthatch) of *Ae. aegypti* was more than a two-fold higher than that found in older adults (7-d-old female, i.e., 19 d posthatch and 14-d-old female, i.e., 26 d posthatch) of *Ae. aegypti* (Fig. 1). Relative expression of *AeaCytC* in female mosquitoes was not significantly different between the 7- (43 ± 1.18) and 14-d-old (40 ± 1.24) mosquitoes.

AeaCYTC Protein Levels in Different Developmental Stages of *Ae. aegypti*. To understand whether the levels of AeaCYTC protein differed by developmental stages, we used Western blot analysis to examine differential protein expressions from *Ae. aegypti* eggs, larvae, pupae, and adults. Western blot data showed that AeaCYTC protein was only detectable in adult mosquitoes (Fig. 2; Table 1). AeaCYTC protein in young adults (2-d old) was ≈10-fold less than that in 10-d-old adults of *Ae. aegypti* (Table 1). Western blot analyses revealed that AeaCYTC protein expression increased with age, especially during the first 2–3 d after adult emergence. Therefore, our data demonstrated that AeaCYTC protein level was regulated and differentially expressed within the developmental stages.

Effects of UV-Light on AeaCytC and AeaCYTC Expression in Female Adult *Ae. aegypti*. In nature, mosquitoes live in various environments. To understand extreme environmental effects on mosquito survival and cytochrome *c* regulation, we designed UV-light and heat-shock experiments to examine *AeaCytC* gene expression levels by examining adults of different ages.

AeaCytC gene expression increased dramatically in 3- and 10-d-old adults exposed to UV-light. This in-

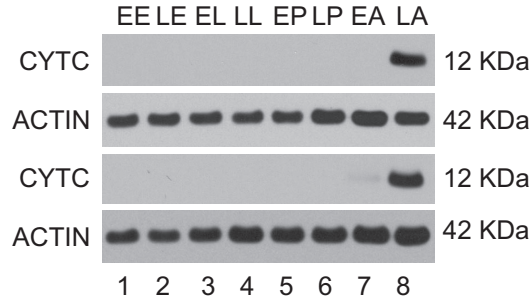

Fig. 2. Western blots showing that the cytochrome *c* protein (AeaCYTC) can only be detected in the adult mosquitoes. Lane 1, early egg (EE); lane 2, late egg (LE); lane 3, first instar (EL); lane 4, fourth instar (LL); lane 5, early pupae (EP); lane 6, late pupae (LP); lane 7, first two panels are teneral adults; third and fourth panels are 2-d-old adults (early adults, EA); and lane 8, first two panels are 8-d-old adults; third and fourth panels are10-d-old adults (late adults, LA).

Table 1. Average pixel density of AeaCYTC expression from Western blots in Fig. 2

Blot lane[a]	1	2	3	4	5	6	7	8
CYTC	7.3	8.1	7.3	7.6	7.4	7.9	8.6	158
ACTIN	189.5	196.7	194.3	182.7	185.7	226.9	239.9	222.1
CYTC	7.9	7.7	7.1	7.0	6.8	7.2	17.8	162.8
ACTIN	146.4	134.6	156.4	189.2	177.2	187.4	206.4	202.9

[a] Blot lanes correspond to Fig. 2.

creased expression occurred at the 15-min exposure, and then it decreased at 30 min and 3-h exposure (Fig. 3A). For 3-d-old adult mosquitoes, relative *AeaCytC* gene expression levels increased from 0 min (3.8 ± 0.09) to 15 min (11.77 ± 0.71). This increase also was observed in 10-d-old adults (Fig. 3A), i.e., from 0- (1.69 ± 0.12) to 15-min (8.3 ± 0.43) exposure. However, the expression level in 17-d-old adults changed very little at each time of UV-treatment (Fig. 3A). The data demonstrated that various ages of *Ae. aegypti* adults have different sensitivities to UV-light exposure.

The AeaCYTC protein expression level at 3-h UV-treated 3-d-old adult mosquitoes was significantly reduced compared with that in untreated mosquitoes (Fig. 3B; Table 2). However, the protein level with 3-h UV-treatment in 10-d-old *Ae. aegypti* remained unchanged.

Effect of Temperatures on AeaCytC and AeaCYTC Expression in Adult *Ae. aegypti*. To evaluate the age-related response of environmental temperatures to cytochrome *c* regulation in adult *Ae. aegypti*, we studied gene expression response to heat stress in both young and old adults. RT-PCR data indicated that *AeaCytC* gene expression was elevated after 15 min of heat exposure in both young (2-d-old) and older (9-d-old) adults (Fig. 4A and B). However, after 30 and 180 min of heat exposure, *AeaCytC* gene expression was dramatically reduced (Fig. 4, A and B). In addition, different temperatures had age-related effect on *AeaCytC* gene expression. Two-day-old adults had lower *AeaCytC* gene expression (after 30-min heat treatment) at both 37 and 40°C compared with that at 24°C. In contrast, 9-d-old adults had higher *AeaCytC* gene expression (after 30-min heat treatment) at 37 and 40°C compared with that at 24°C. This indicated that environmental temperatures affected *AeaCytC* gene expression and had the greatest effect at the initial exposure time point.

In 2-d-old *Ae. aegypti* adults, AeaCYTC protein expression increased at the 180-min post treatment at both 37°C (lane 6) and 40°C (lane 9) (Fig. 4C; Table 3). However, 9-d-old *Ae. aegypti* adults, 180 min of heat treatment (lanes 6 and 9, third panel) did not change

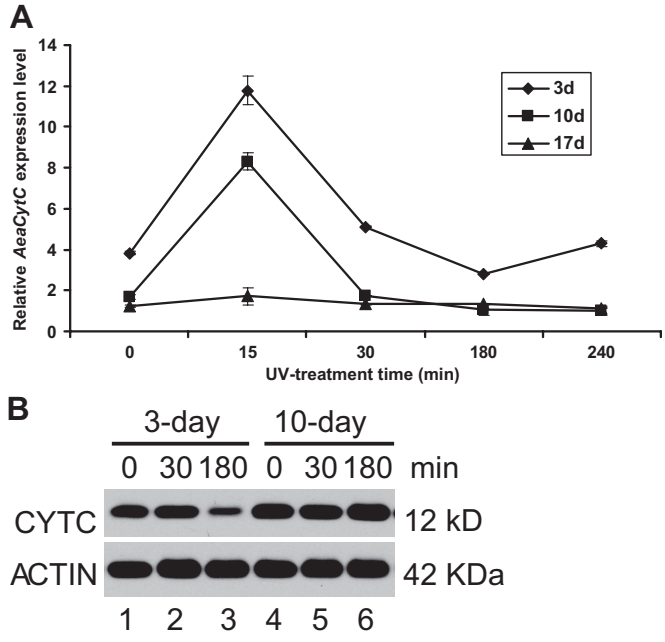

Fig. 3. (A) Time course of *AeaCytC* expressed in *Ae. aegypti* adults of different ages after UV-light treatment. Three-, 10-, and 17-d-old mosquitoes were exposed to UV-light for 0, 15, 30, 180, and 240 min. Please note x-axis is not to scale. (B) Western blot analysis for 3- and 10-d-old adult *Ae. aegypti* with UV-light treatment. Lanes 1–3, 3-d-olds; lanes 4–6, 10-d-olds. 1 and 4, control, 0-min UV-treatment; 2 and 5, 30-min UV-treatment; and 3 and 6, 180-min UV-treatment.

Table 2. Average pixel density of AeaCYTC expression from Western blots in Fig. 3B

Blot lane[a]	1	2	3	4	5	6
CYTC	145.9	148.1	76.9	162.8	161.1	180.9
ACTIN	181.4	200.4	193.7	188.7	188.9	188.4

[a] Blot lanes correspond to Fig. 3B.

AeaCYTC protein levels (Fig. 4C; Table 3). These results indicated that AeaCYTC protein function might have decreased with age in adult mosquitoes in response to heat shock.

Effects of Permethrin on AeaCytC and AeaCYTC Expression in Adult *Ae. aegypti*. To determine whether the expression of *AeaCytC* gene in *Ae. aegypti* was affected by permethrin treatment, mosquitoes of different ages were treated with different concentrations of permethrin as described previously using acetone as a carrier (Pridgeon et al. 2007). Quantitative real-time PCR data showed that permethrin-treated mosquitoes had decreased *AeaCytC* expression at 1 h posttreatment compared with treatment with acetone only as a control (Fig. 5A and B). Student's *t*-test (Steel et al. 1998) showed significantly difference relative levels of *AeaCytC* expression at 1 h posttreatment among the permethrin and acetone treatments, with a criterion of $P \leq 0.05$. Permethrin-treated *Ae. aegypti* adults continued to show a decrease in *AeaCytC* gene expression until 12 h postexposure.

In 3-d-old *Ae. aegypti* adults, AeaCYTC protein expression decreased slightly as the acetone treatment time increased from 6 to12 h (lanes 4 and 5) (Fig. 5C; Table 4). In 3-d-old *Ae. aegypti* adults, AeaCYTC protein expression increased little as postpermethrin treatment time increased from 6 to 12 h (lanes 9 and 10) (Fig. 5C; Table 4). However, in 10-d-old *Ae. aegypti* adults, AeaCYTC protein expression decreased 10–15% as postpermethrin treatment time increased from 6 to 12 h (lanes 9 and 10, third panel) (Fig. 5C; Table 4). Acetone treated 10-d-old *Ae. aegypti* adults showed AeaCYTC protein expression levels similar to that of 3-d-old adults and expression decreased slightly as the acetone treatment time increased from 6 to12 h (lanes 4 and 5, third panel) (Fig. 5C; Table 4). These results demonstrated that AeaCYTC protein function decreased with age in adult mosquitoes in response to permethrin treatment.

Discussion

Cytochrome *c* Functions during *Ae. aegypti* Development. We analyzed changes in the gene and protein expression of cytochrome *c* in *Ae. aegypti* from eggs, larvae, pupae, and adult females. There were signifi-

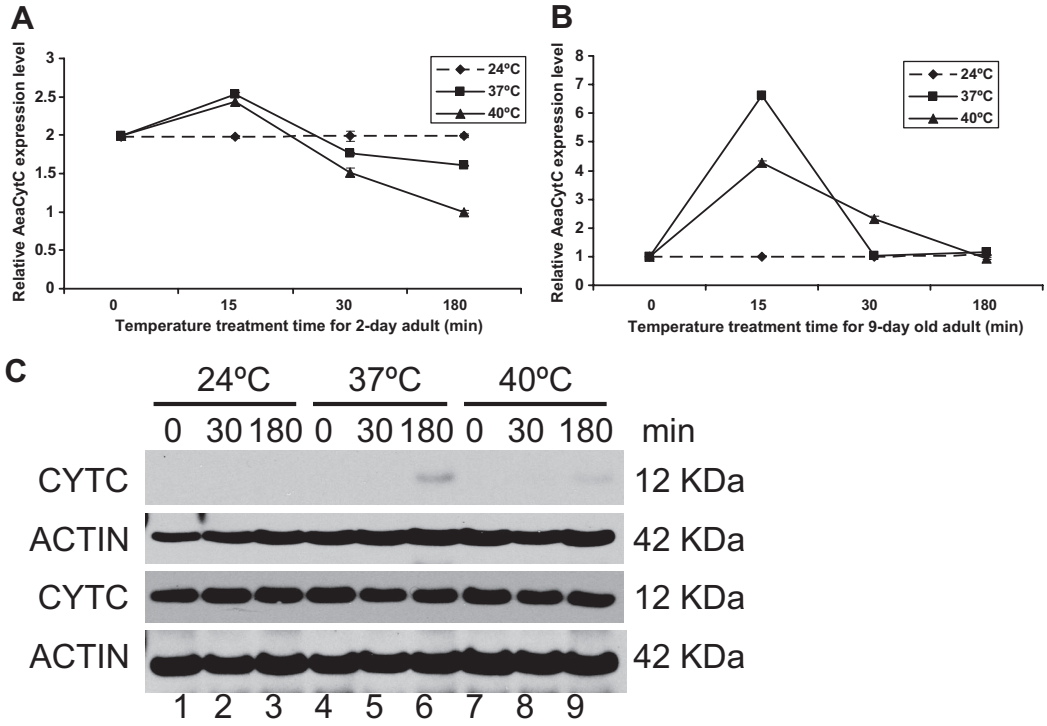

Fig. 4. (A and B) Quantitative real-time PCR time course of *AeaCytC* expressed in 2- and 9-d-old adult *Ae. aegypti* exposed to different temperatures. Please note x-axis is not to scale. (A) 2-d-old adults exposed at 24, 37, and 40°C. (B) Nine-day-old adults exposed to 24, 37, and 40°C. (C) Western blot analysis showing AeaCYTC protein expression in 2- and 9-d-old adults of *Ae. aegypti* after a 3-h heat treatment. Lanes 1–3, 24°C; lanes 4–6, 37°C; lanes 7–9, 40°C. Lanes 1, 4, and 7, 0 min; lanes 2, 5, and 8, 30 min; lanes 3, 6, and 9, 180 min. First two panels are 2-d-old adults; third and fourth panels are 9-d-old adults.

Table 3. Average pixel density of AeaCYTC expression from Western blots in Fig. 4C

Blot lane[a]	1	2	3	4	5	6	7	8	9
CYTC (2 d)[b]	52.2	55.7	53.1	52.6	56.3	150.1	53.8	58.7	88.8
ACTIN (2 d)	173.3	195.4	213.1	217.9	213.4	213.6	213.8	214.5	211.4
CYTC (9 d)[c]	172.7	207.5	203.3	209.3	174.1	180.2	203.1	177.2	186.4
ACTIN (9 d)	220.7	212.4	217.9	217.7	227.3	231.5	236.4	235.8	228.6

[a] Blot lanes correspond to Fig. 4C.
[b] CYTC expression in 2-d-old adult *Ae. aegypti.*
[c] CYTC expression in 9-d-old adult *Ae. aegypti.*

cant differences in the expression of *AeaCytC* genes between young (94.37 ± 1.34) and old (40.10 ± 1.24) adult mosquitoes according to Student's *t*-test (Steel et al. 1998), with a criterion of $P \leq 0.05$. Numerous physiological changes occur during the development of female adult mosquitoes as they age. The *AeaCytC* gene is highly expressed in teneral adults, which may indicate that mitochondrial activity at this time is high as adults prepare for a bloodmeal and ovarian development. However, levels of AeaCYTC protein were even higher in 9- and 14-d-old adults than in 1- and 2-d-old adults of *Ae. aegypti,* suggesting that cytochrome *c* protein expression in mature mosquitoes is critical for mitochondrial functions and that it may be related to mosquito aging. In addition, the *AeaCytC* gene expression and AeaCYTC protein levels during the mosquito development were not identical at the same point in time, which indicated that gene regulation and protein function of cytochrome *c* is a complex process in adult mosquito development.

The study of age-related gene expression is critical for a better understanding of physiological changes during aging. An age-associated decline in the immunological capabilities of mosquitoes after challenge with an infectious agent has been reported previously (Hillyer et al. 2005). The relative low levels of *AeaCytC* gene expression in older or senescent mosquitoes suggest that mitochondrial dysfunction may play a role in the attenuation of gene expression.

Mosquito development can be regulated by multigenes (Severson et al. 2004, Fontenille et al. 2005, Raibaud et al. 2006, Strode et al. 2006, van den Hurk

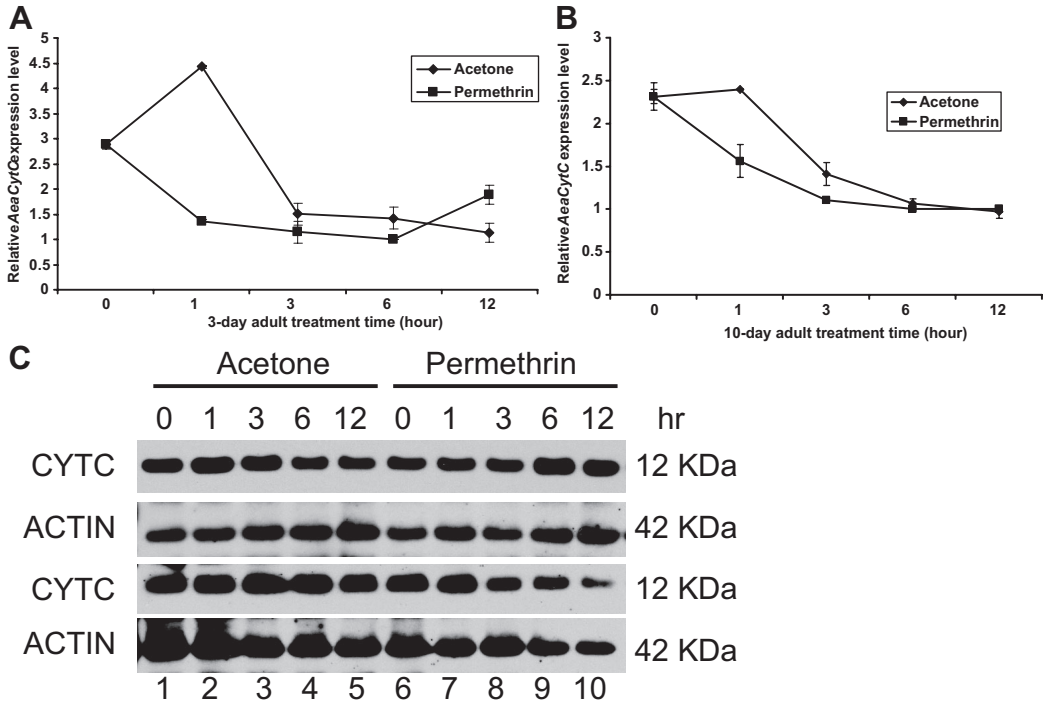

Fig. 5. (A and B) Quantitative real-time PCR time course of *AeaCytC* expressed in 3- and 9-d-old *Ae. aegypti* adults after acetone and permethrin (1.25×10^{-5} μg per mosquito) treatments. Please note x-axis is not to scale. (C) Western blot analysis of AeaCYTC protein expression in 3- and 10-d-old adults of *Ae. Aegypti* after acetone and permethrin (1.25×10^{-5} μg per mosquito) treatments. Lanes 1–5, acetone treatment; lanes 6–10, permethrin treatment. Lanes 1 and 6, 0 h after treatment; lanes 2 and 7, 1 h after treatment; lanes 3 and 8, 3 h after treatment; lanes 4 and 9, 6 h after treatment; lanes 5 and, 10–12 h after treatment. First two panels are 3-d-old adults; third and fourth panels are 10-d-old adults.

Table 4. Average pixel density of AeaCYTC expression from Western blots in Fig. 5C

Blot lane[a]	1	2	3	4	5	6	7	8	9	10
CYTC (3d)[b]	213.6	227.9	217.8	189.7	188.9	195.6	195.7	192.7	226.4	208.2
ACTIN (3d)	209.9	215.4	223.8	226.0	230.5	209.3	223.6	206.7	226.3	229.0
CYTC (10d)[c]	208.5	221.6	223.4	216.9	198.5	205.1	202.3	169.8	166.4	133.2
ACTIN (10d)	232.3	231.8	225.7	219.3	221.4	223.9	211.7	220.4	195.3	174.6

[a] Blot lanes correspond to Fig. 5C.
[b] CYTC expression in 3-d-old adult *Ae. aegypti.*
[c] CYTC expression in 10-d-old adult *Ae. aegypti.*

et al. 2007). Cytochrome *c* might be one of these genes. Teneral *Ae. aegypti* adults may have less developed mitochondria that may explain why AeaCYTC protein expression is low. As adult mosquitoes age (>3 d old), AeaCYTC protein expression is enhanced, possibly indicating that mitochondria in the adult mosquito have completed development in preparation for the first bloodmeal.

Cytochrome *c* Responses to Environmental Stresses. The *AeaCytC* gene of *Ae. aegypti* responded differently to challenge by heat stress and UV-radiation. Temperature affects biochemical, physiological, and behavioral processes in mosquitoes (Narang and Narang 1975, Beach et al. 1989, Mahmood and Crans 1997, Wiwatanaratanabutr and Kittayapong 2006). In our study, the influence of heat stress on the expression of the *AeaCytC* gene was shown to be higher in 2-d-old mosquitoes than in 9-d-old mosquitoes. The time course study of *AeaCytC* gene expression in *Ae. aegypti* of different ages suggests that *AeaCytC* is regulated by temperature. Heat stress treatments induce activation of AeaCYTC protein only in 2-d-old adults but not in 9-d-old adults of *Ae. aegypti.* The present results demonstrate the lack of response to heat stress, or even the attenuation of gene expression, as mosquitoes become older, which may play an important role in age-related physiological changes.

UV-radiation is known to affect the integrity of cuticle in adult mosquitoes (Sugumaran et al. 1992). In a previous study on adults of *Anopheles gambiae* (Giles), there were quantitative differences in 48 cuticular hydrocarbons between 0–2 d and older invididuals (Caputo et al. 2005). Protein expression levels in 3-d-old *Ae. aegypti* after a 3-h UV-treatment (lane 3) was significantly reduced (Fig. 3B). However, the protein level in 10-d-old *Ae. aegypti* after a 3-h UV-treatment (lane 6) remained unchanged. This result may indicate that older mosquitoes have more protection (are less sensitive) to UV-light, possibly due to changes in cuticle composition that occurs with age.

Effect of Permethrin on Cytochrome *c* Gene and Protein Expression. Molecular studies of insecticide resistance have advanced rapidly over the past decade, including identification of genes involved in target site and metabolic resistance mechanisms. The evolution of insecticide resistance acts by selection of these mechanisms, typically requiring the interaction of multiple genes (Liu et al. 2007). The expression of approximately one quarter of the detoxification genes in *An. gambiae* were found to be developmentally regulated (Strode et al. 2006). Our RT-PCR data showed that *AeaCytC* expression levels of 3- and 10-d-old adults of *Ae. aegypti* were down-regulated at 1, 3, and 6 h after permethrin treatment compared with the acetone treatment alone. One hour after acetone treatment, 3-d-old adults of *Ae. aegypti* showed 3 times higher *AeaCytC* expression level than that in the permethrin treatments, whereas 10-d-old adults of *Ae. aegypti* showed only slightly higher *AeaCytC* expression level than that in the permethrin treatments. This indicated that *AeaCytC* gene function and protein levels in adult mosquitoes might have decreased with age in response to the permethrin treatment.

In conclusion, the expressions of *AeaCytC* gene and AeaCYTC protein in the life cycle of *Ae. aegypti* were highly regulated developmentally and environmentally. Cytochrome *c* gene and protein expression has, for the first time, been examined in detail for all developmental stages of *Ae. aegypti.* The current study suggests that *AeaCytC* plays an important role in the development of *Ae. aegypti*, and it may provide information useful for designing novel control strategies for mosquitoes and identifying new pathways to target for the development of molecular pesticides. For example, high levels of *AeaCytC* expression in adult females might direct the design of double-stranded RNA (dsRNA) from *AeaCytC* to knockdown the *AeaCytC* gene, as dsRNA knockdowns have been shown to kill mosquitoes in a previous study (Pridgeon et al. 2008).

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