Expression of *AeaHsp26* and *AeaHsp83* in *Aedes aegypti* (Diptera: Culicidae) Larvae and Pupae in Response to Heat Shock Stress

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ABSTRACT Immature mosquito development and survival of adults are highly sensitive to environmental temperature, which can alter gene expression during the mosquito life-cycle. To further understand how heat shock proteins are developmentally expressed in mosquitoes, we subjected first instar larvae, 16-h old pupae and female of *Aedes aegypti* (L.) (Diptera: Culicidae) to heat shock treatment for 0, 15, 30, 60, and 180 min at 23 and 42°C. The heat shock protein genes *AeaHsp26*, *AeaHsp83*, and *AeaHsc70* were examined by comparing relative transcript expression levels at 42°C compared with 23°C. Upregulated transcripts from heat shock treatment at 42°C and control were further confirmed and quantified by quantitative real-time polymerase chain reaction. Data revealed that first instar larvae were more sensitive to heat shock treatment than pupae and adults (i.e., relative *AeaHsp26* expression levels in larvae were 10-fold greater than in the females. *AeaHsp83* expression levels in larvae, pupae and adults were upregulated 2- to 50-fold greater by heat shock treatment at 42°C compared with 23°C. *AeaHsc70* expression levels in larvae, pupae and adults, however, were upregulated less than *AeaHsp26* and *AeaHsp83* at the higher temperature. Statistical analysis indicated that *AeaHsp26* and *AeaHsp83* genes were significantly upregulated in *Ae. aegypti* larvae and pupae after 15, 30, 60, and 180 min exposure to high temperature (42°C). The current study has shown that *AeaHsp26* and *AeaHsp83* are important markers of stress and may function as critical proteins to protect and enhance survival of *Ae. aegypti* larvae and pupae.

KEY WORDS heat shock, *Aedes aegypti*, gene expression, larvae, development

Temperatures cannot only drastically alter the genetic structure and gene expressions of a vector mosquito population, but can also affect mosquito development (Gakhar and Shandilya 1999, Yadav et al. 2005, Monteiro et al. 2007, Zhao et al. 2009). Several families of heat shock proteins (HSPs) are known to be expressed in insects, including mosquitoes, and may have a cumulative role in responding to stress induced by elevated temperature (Mahroof et al. 2005, Yadav et al. 2005, Rinehart et al. 2006a,b, Robich et al. 2007, Zhao et al. 2009). In addition to heat shock, the expression of HSPs are also induced in response to stress conditions other than temperature in human cells and many other animals and insects including mosquitoes (Mosser et al. 1988, Yamuna et al. 2000, Boone and Vijayan 2002a,b Spees et al. 2002, Cheng et al. 2003, Chen et al. 2005, Hayward et al. 2005, Sim et al. 2005, Chuang et al. 2007, Sim et al. 2007). Therefore, the expression of HSPs in different developmental stages is important to understand the overall response of mosquitoes to heat shock and other types of environmental stress.

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Previous work using subtracted cDNA libraries has shown that *AeaHsp26*, *AeaHsp83*, and *AeaHsc70* are differentially expressed in the adult *Aedes aegypti* (L.) (Zhao et al. 2009). To better understand HSP family genes expressed in first instar larvae, pupae and female *Ae. aegypti*, we used quantitative real-time polymerase chain reaction (qPCR) to examine *AeaHsp26*, *AeaHsp83*, and *AeaHsc70* expression levels in response to heat shock. As part of our effort to develop genetic and molecular mechanism for mosquito control, the overexpression of HSP genes in larvae, pupae and adults may provide information needed to identify HSP proteins critical to mosquito survival (Zhao et al. 2009).

Materials and Methods

Mosquito Strains. *Ae. aegypti* (Orlando, FL, strain, maintained since 1952) were reared in the insectary of the Mosquito and Fly Research Unit at the Center for Medical, Agricultural, and Veterinary Entomology, USDA-ARS, Gainesville, FL.

Heat-Shock Experiments. To test how high temperatures affect gene expression, extreme heat shock treatment (42°C) of larvae, pupae and females were conducted to optimize the response and facilitate detection of the differentially expressed genes. First in-

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14. ABSTRACT

Immature mosquito development and survival of adults are highly sensitive to environmental temperature, which can alter gene expression during the mosquito life-cycle. To further understand how heat shock proteins are developmentally expressed in mosquitoes, we subjected ?st instar larvae, 16-h old pupae and female of Aedes aegypti (L.) (Diptera: Culicidae) to heat shock treatment for 0, 15, 30, 60, and 180 min at 23 and 42 C. The heat shock protein genes AeaHsp26 AeaHsp83, and AeaHsc70 were examined by comparing relative transcript expression levels at 42 C compared with 23 C. Upregulated transcripts from heat shock treatment at 42 C and control were further con?med and quanti?d by quantitative real-time polymerase chain reaction. Data revealed that ?st instar larvae were more sensitive to heat shock treatment than pupae and adults (i.e., relative AeaHsp26 expression levels in larvae were 10-fold greater than in the females. AeaHsp83 expression levels in larvae, pupae and adults were upregulated 2- to 50-fold greater by heat shock treatment at 42 C compared with 23 C. AeaHsc70 expression levels in larvae, pupae and adults, however, were upregulated less than AeaHsp26 and AeaHsp83 at the higher temperature. Statistical analysis indicated that AeaHsp26 and AeaHsp83 genes were signi?antly upregulated in Ae. aegypti larvae and pupae after 15, 30, 60, and 180 min exposure to high temperature (42 C). The current study has shown that AeaHsp26 and AeaHsp83 are important markers of stress andmayfunction as critical proteins to protect and enhance survival of Ae. aegypti larvae and pupae.

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Table 1. Primers used for qPCR

Primer name	Sequence
AeaHsp26-F	5′- TTCAGCATCCTTCTCCTCGT-3′
AeaHsp26-R	5′-CCACGAACTTCCAGGTCAAT-3′
AeaHsp83-F	5′- AAGGCCGTTAAGGATCTGGT-3′
AeaHsp83-R	5′- CGCTAGTGTGGGGAAGAGAG-3′
AeaHsc70-F	5′- ATGAACCCAACCAACACCAT-3′
AeaHsc70-R	5′-TGGAACTGATTTCCTCTGGG-3′
AeaActin-F	5′- AGGACTCGTACGTCGGTGAC-3′
AeaActin-R	5′- CGTTCAGTCAGGATCTTC-3′

star larvae, young pupae (<16 h) and 7-d-old females *Ae. aegypti* were exposed to two different temperatures (23 and 42°C) and held in an environmental chamber (L-C Incubator, Lab-Line Instruments, Inc., Melrose Park, IL) for the time course study. Specifically, first instar larvae and pupae in deionized water (DI H_2O) were exposed in an environmental chamber to 42°C for 0, 15, 30, 60, and 180 min. Untreated larvae and pupae (controls) were held at constant room temperature (23°C) in DI H_2O, while a cage of females was held at constant room temperature (23°C) and in an incubator at 42°C for the five time points indicated above. Three replicates were performed for each experiment. We collected 100 μg of larvae, 10 pupae and 10 adults for each RNA extraction sample.

RNA Extraction and cDNA Synthesis. Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Poly(A)$^+$ RNA was isolated by applying Oligotex-dT suspension (QIAGEN, Valencia, CA). RNA samples were quantified by SmartSpec Plus Spectrophotometry (BIO-RAD, Hercules, CA). A 3-μg aliquot of purified RNA was reverse transcribed in 20-μl reaction volume using Cloned AMV First-Strand Synthesis Kit for RT-PCR according to the manufacturer's instructions (Invitrogen). The reaction was terminated by heat inactivation at 95°C for 5 min. The cDNA samples for heat shock treatment and control were diluted by adding 80 μl ddH_2O (300 ng/μl) and stored at −20°C.

Quantitative qPCR Amplification. The qPCR assay for HSP genes was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) in a volume of 15 μl on an Applied Biosystems 7300 Fast Real-Time PCR System (Foster City, CA). The PCR mixture consisted of 1 μl diluted cDNA (300 ng/μl), 0.5 μM primers, and 1X master mix. In all qPCR runs, *AeaActin* was used as an internal control to normalize for variation in the amount of cDNA template. The PCR primers used were *Aea-Gene-F* and *Aea-Gene-R* including control PCR primers for *Aea-Actin* (Table 1). There are three HSP genes in *Ae.*

Table 2. Expression of *AeaHsp26* genes under heat shock treatment in *Aedes aegypti*

Time point	Cycle threshold (Ct) ± SD		Relative *AeaHsp26* expression level			
	Actin	*AeaHsp26*	ΔΔCt-1[a]	ΔΔCt-2[a]	ΔΔCt-3[a]	$2^{--\Delta\Delta Ct}$ ± SD
23°C — 0′[b]	15.915 ± 0.197	23.352 ± 0.010	—	—	—	—
23°C — 15′[b]	15.816 ± 0.007	25.589 ± 0.078	2.273	2.394	2.334	0.1985 ± 0.008
23°C — 30′[b]	15.434 ± 0.022	25.594 ± 0.121	2.824	2.621	2.723	0.1518 ± 0.011
23°C — 60′[b]	15.089 ± 0.024	24.529 ± 0.025	1.967	2.037	2.002	0.2498 ± 0.006
23°C — 180′[b]	14.521 ± 0.075	25.786 ± 0.170	3.760	3.895	3.828	0.0705 ± 0.003
42°C — 0′[b]	15.915 ± 0.197	23.352 ± 0.010	—	—	—	—
42°C — 15′[b]	18.314 ± 0.020	17.648 ± 0.036	−8.092	−8.115	−8.103	275.03 ± 2.154
42°C — 30′[b]	16.129 ± 0.061	15.360 ± 0.148	−8.059	−8.354	−8.207	295.43 ± 30.31
42°C — 60′[b]	16.228 ± 0.040	14.306 ± 0.023	−9.405	−9.315	−9.360	657.42 ± 20.41
42°C — 180′[b]	14.769 ± 0.019	14.437 ± 0.043	−7.786	−7.753	−7.769	218.22 ± 2.511
23°C — 0′[c]	12.498 ± 0.141	21.747 ± 0.351	—	—	—	—
23°C — 15′[c]	12.487 ± 0.038	20.265 ± 0.057	−1.539	−1.404	−1.471	2.773 ± 0.183
23°C — 30′[c]	12.770 ± 0.165	19.791 ± 0.014	−2.121	−2.335	−2.228	4.684 ± 0.492
23°C — 60′[c]	12.767 ± 0.268	19.364 ± 0.096	−2.395	−2.909	−2.652	6.288 ± 1.594
23°C — 180′[c]	13.322 ± 0.701	19.538 ± 0.268	−2.728	−3.340	−3.034	8.189 ± 2.473
42°C — 0′[c]	12.969 ± 0.125	20.554 ± 0.073	—	—	—	—
42°C — 15′[c]	12.331 ± 0.192	13.262 ± 0.087	−8.515	−8.392	−8.453	350.52 ± 21.24
42°C — 30′[c]	12.663 ± 0.029	12.762 ± 0.269	−8.873	−8.980	−8.927	486.65 ± 25.53
42°C — 60′[c]	13.179 ± 0.143	12.567 ± 0.133	−9.853	−9.868	−9.860	929.46 ± 6.742
42°C — 180′[c]	12.856 ± 0.036	13.689 ± 0.096	−8.457	−8.373	−8.415	341.28 ± 14.10
23°C — 0′[d]	16.241 ± 0.121	23.639 ± 0.145	—	—	—	—
23°C — 15′[d]	16.386 ± 0.040	22.452 ± 0.066	−1.351	−1.314	−1.332	2.518 ± 0.045
23°C — 30′[d]	15.471 ± 0.050	24.516 ± 0.027	1.663	1.631	1.647	0.319 ± 0.005
23°C — 60′[d]	16.053 ± 0.016	23.475 ± 0.035	0.010	0.036	0.023	0.984 ± 0.013
23°C — 180′[d]	15.544 ± 0.025	22.743 ± 0.042	−0.212	−0.188	−0.199	1.149 ± 0.013
42°C — 0′[d]	15.868 ± 0.030	22.337 ± 0.050	—	—	—	—
42°C — 15′[d]	16.189 ± 0.007	18.873 ± 0.179	−3.668	−3.932	−3.799	13.927 ± 1.808
42°C — 30′[d]	16.270 ± 0.013	16.723 ± 0.030	−6.018	−6.042	−6.030	65.352 ± 0.765
42°C — 60′[d]	17.172 ± 0.043	18.414 ± 0.022	−5.226	−5.256	−5.241	37.809 ± 0.554
42°C — 180′[d]	15.802 ± 0.039	16.301 ± 0.048	−5.981	−5.969	−5.975	62.901 ± 0.382

[a] Example of relative *AeaHsp26*ΔCt calculation using *Actin* as reference gene. Relative *AeaHsp26* $\Delta\Delta C_t$ at a time point = *AeaHsp26*ΔC_t–*AeaHsp26*ΔC_t-0 at 0 h point.

[b] First instar larvae.

[c] Pupae (<16 h).

[d] Seven day-old female.

Table 3. Expression of *AeaHsp83* genes under heat shock treatment in *Aedes aegypti*

Time point	Cycle threshold (Ct) ± SD		Relative *AeaHsp83* expression level			
	Actin	*AeaHsp83*	$\Delta\Delta C_t$-1[a]	$\Delta\Delta C_t$-2[a]	$\Delta\Delta C_t$-3[a]	$2^{--\Delta\Delta Ct}$ ± SD
23°C — 0′[b]	15.915 ± 0.197	16.677 ± 0.070	—	—	—	—
23°C — 15′[b]	15.816 ± 0.007	16.206 ± 0.018	−0.364	−0.381	−0.373	1.295 ± 0.010
23°C — 30′[b]	15.434 ± 0.022	15.860 ± 0.055	−0.360	−0.312	−0.336	1.262 ± 0.030
23°C — 60′[b]	15.089 ± 0.024	16.978 ± 0.049	1.075	1.178	1.127	0.458 ± 0.023
23°C — 180′[b]	14.521 ± 0.075	17.962 ± 0.071	2.783	2.577	2.679	0.156 ± 0.016
42°C — 0′[b]	17.003 ± 0.118	18.629 ± 0.046	—	—	—	—
42°C — 15′[b]	18.314 ± 0.020	16.327 ± 0.025	−2.786	−2.716	−2.74	6.719 ± 0.212
42°C — 30′[b]	16.129 ± 0.061	13.355 ± 0.002	−3.494	−3.578	−3.537	11.61 ± 0.477
42°C — 60′[b]	16.228 ± 0.040	12.579 ± 0.032	−4.417	−4.406	−4.412	21.28 ± 0.119
42°C — 180′[b]	14.769 ± 0.019	12.479 ± 0.237	−3.206	−2.899	−3.053	8.296 ± 1.254
23°C — 0′[c]	13.313 ± 0.193	15.154 ± 0.026	—	—	—	—
23°C — 15′[c]	13.040 ± 0.022	14.254 ± 0.085	−0.672	−0.582	−0.627	1.5442 ± 0.675
23°C — 30′[c]	12.668 ± 0.103	13.750 ± 0.099	−0.757	−0.763	−0.759	1.6934 ± 0.005
23°C — 60′[c]	13.048 ± 0.010	14.257 ± 0.017	−0.651	−0.612	−0.631	1.5282 ± 0.030
23°C — 180′[c]	13.076 ± 0.089	14.626 ± 0.127	−0.317	−0.264	−0.291	1.2233 ± 0.032
42°C — 0′[c]	13.190 ± 0.036	15.139 ± 0.034	—	—	—	—
42°C — 15′[c]	13.226 ± 0.022	12.348 ± 0.037	−2.761	−2.677	−2.719	6.5839 ± 0.269
42°C — 30′[c]	12.838 ± 0.112	11.033 ± 0.044	−3.597	−3.694	−3.645	12.513 ± 0.596
42°C — 60′[c]	13.442 ± 0.054	11.007 ± 0.113	−4.318	−4.234	−4.276	19.369 ± 0.800
42°C — 180′[c]	11.843 ± 0.059	10.687 ± 0.037	−2.929	−3.066	−2.998	7.9881 ± 0.532
23°C — 0′[d]	16.241 ± 0.121	16.458 ± 0.051	—	—	—	—
23°C — 15′[d]	16.386 ± 0.040	16.561 ± 0.034	0.011	−0.095	−0.042	1.029 ± 0.053
23°C — 30′[d]	15.471 ± 0.050	16.111 ± 0.024	0.476	0.371	0.423	0.745 ± 0.038
23°C — 60′[d]	16.053 ± 0.016	16.793 ± 0.071	0.484	0.561	0.523	0.696 ± 0.026
23°C — 180′[d]	15.544 ± 0.025	16.238 ± 0.120	0.411	0.545	0.478	0.718 ± 0.047
42°C — 0′[d]	15.868 ± 0.030	16.704 ± 0.049	—	—	—	—
42°C — 15′[d]	16.189 ± 0.007	15.696 ± 0.059	−1.296	−1.389	−1.343	2.535 ± 0.116
42°C — 30′[d]	16.270 ± 0.013	14.531 ± 0.037	−2.624	−2.552	−2.588	6.013 ± 0.212
42°C — 60′[d]	17.172 ± 0.043	15.134 ± 0.012	−2.866	−2.909	−2.887	7.398 ± 0.158
42°C — 180′[d]	15.802 ± 0.039	13.398 ± 0.112	−3.306	−3.202	−3.254	9.538 ± 0.485

[a] Example of relative *AeaHsp83* ΔCt calculation using *Actin* as reference gene. Relative *AeaHsp83* $\Delta\Delta C_t$ at a time point = *AeaHsp83*ΔC_t–*AeaHsp83*ΔC_t-0 at 0 h point.
[b] First instar larvae.
[c] Pupae (<16 h).
[d] Seven day-old female.

aegypti: (1) *AeaHsp26*, a small heat-shock protein; (2) *AeaHsp83*, the expression of *Hsp90* protein, encoded by the *AeaHsp83;* and (3) *AeaHsc70*, *Hsc70.*

The PCR thermal cycling parameters were the same as described previously (Zhao et al. 2008). Relative expression levels were calculated as follows: first, *AeaHsp* transcript levels relative to a standard (*AeaActin*) were found using the formula $\Delta C_T = C_T$ (*AeaHsp*) - C_T (*AeaActin*). Second, a $\Delta\Delta C_T$ value for each sample was calculated by subtracting the 0 min control $\Delta\Delta C_T = \Delta C_T - \Delta C_{T=0}$. Third, relative expression levels were calculated using the modified equation 1 by $2^{-[\text{average } \Delta\Delta CT\ ]}$ (Portereiko et al. 2006).

Statistical Analysis. Comparisons of means were analyzed using the Student's *t*-test (Steel et al. 1998), and *t*-values and *P* values were reported when normality and equal variance tests were passed. Significant differences between the data were determined using SigmaStat software (SigmaStat 3.5, Systat Software, Inc., San Jose, CA).

Results

High Temperature Effects on Relative RNA Expression Levels of *AeaHsp26*, *AeaHsp83*, and *AeaHsc70* in *Ae. aegypti* Larvae. To determine whether HSP genes that are differentially expressed during heat shock treatment in *Ae. aegypti* adults (Zhao et al. 2009) are also overexpressed in larvae by exposure to high temperature conditions, qPCR analyses were performed on *Ae. aegypti* larvae exposed to heat shock (42°C) and at room temperature (23°C) over time. Three genes from the HSP gene family (*AeaHsp26*, *AeaHsp83*, and *AeaHsc70*) were examined after heat shock treatment (Tables 2-4). According to our qPCR data, HSP genes found in the subtraction cDNA library in adult *Ae. aegypti* (Zhao et al. 2009) were also up-regulated in larval *Ae. aegypti* (Tables 2–4). The RNA relative gene expression level of *AeaHsp26* and *AeaHsp83* increased significantly after 42°C treatment of *Ae. aegypti* larvae for 15 min compared with the control (Tables 2 and 3, Figs. 1A and 2A, supplementary data Table 5 and 6). For example, the relative expression of a small HSP *AeaHsp26* was up-regulated after 15 min at 42°C treatment of first instar *Ae. aegypti* larvae (275.03 ± 2.154), more than a 1,300-fold increase over that found in the untreated control (0.1985 ± 0.008) (Table 2, Fig. 1A). As the time of heat shock treatment increased (30, 60, and 180 min), the relative gene expression level of *AeaHsp26* in larval *Ae. aegypti* also increased and reached ≈3,000-fold after 3 h at 42°C treatment compared with the control (Table 2, Fig. 1A).

The expression of *Hsp90* protein, encoded by *AeaHsp83* (83 kDa) was up-regulated after 1 h at 42°C

Table 4. Expression of *AeaHsc70* genes under heat shock treatment in *Aedes aegypti*

Time point	Cycle threshold (Ct) ± SD		Relative *AeaHsc70* expression level			
	Actin	*AeaHsc70*	ΔΔCt-1[a]	ΔΔCt-2[a]	ΔΔCt-3[a]	$2^{--\Delta\Delta Ct}$ ± SD
23°C — 0′[b]	14.433 ± 0.169	13.321 ± 0.061	—	—	—	—
23°C — 15′[b]	20.135 ± 0.692	19.923 ± 0.189	0.5448	0.900	1.256	0.5357 ± 0.189
23°C — 30′[b]	16.039 ± 0.123	14.822 ± 0.015	−0.007	−0.203	−0.105	1.0755 ± 0.104
23°C — 60′[b]	17.482 ± 0.449	16.144 ± 0.081	−0.599	−0.666	−0.225	1.1689 ± 0.050
23°C — 180′[b]	14.610 ± 0.085	14.149 ± 0.042	0.561	0.741	0.651	0.6368 ± 0.056
42°C — 0′[b]	14.438 ± 0.231	14.435 ± 0.085	—	—	—	—
42°C — 15′[b]	14.122 ± 0.535	13.027 ± 0.103	−1.544	−0.641	−1.092	2.1323 ± 0.959
42°C — 30′[b]	17.247 ± 0.078	16.171 ± 0.006	−1.131	−1.013	−1.072	2.1023 ± 0.123
42°C — 60′[b]	15.271 ± 0.159	14.815 ± 0.009	−0.335	−0.573	−0.454	1.3699 ± 0.160
42°C — 180′[b]	15.092 ± 0.055	13.707 ± 0.014	−1.332	−1.430	−1.382	2.6056 ± 0.125
23°C — 0′[c]	12.498 ± 0.141	13.315 ± 0.145	—	—	—	—
23°C — 15′[c]	12.487 ± 0.038	13.241 ± 0.048	−0.133	−0.011	−0.072	1.051 ± 0.063
23°C — 30′[c]	12.770 ± 0.165	13.567 ± 0.076	0.035	−0.093	−0.029	1.020 ± 0.064
23°C — 60′[c]	12.767 ± 0.268	14.278 ± 0.130	0.782	0.587	0.684	0.622 ± 0.059
23°C — 180′[c]	13.322 ± 0.701	14.435 ± 0.050	0.254	0.324	0.289	0.818 ± 0.028
42°C — 0′[c]	12.969 ± 0.125	14.780 ± 0.219	—	—	—	—
42°C — 15′[c]	12.331 ± 0.192	14.287 ± 0.124	−0.077	0.099	0.011	0.993 ± 0.085
42°C — 30′[c]	12.663 ± 0.029	14.068 ± 0.017	0.050	−0.413	−0.181	1.134 ± 0.259
42°C — 60′[c]	13.179 ± 0.143	13.772 ± 0.096	−1.183	−1.249	−1.216	2.323 ± 0.076
42°C — 180′[c]	12.856 ± 0.036	12.585 ± 0.115	−2.136	−2.024	−2.080	4.228 ± 0.232
23°C — 0′[d]	14.154 ± 0.178	14.368 ± 0.042	—	—	—	—
23°C — 15′[d]	14.332 ± 0.078	14.716 ± 0.009	0.108	0.232	0.169	0.889 ± 0.054
23°C — 30′[d]	13.295 ± 0.038	13.542 ± 0.081	0.004	0.063	0.034	0.976 ± 0.028
23°C — 60′[d]	13.659 ± 0.205	14.487 ± 0.067	0.421	0.806	0.614	0.653 ± 0.124
23°C — 180′[d]	13.345 ± 0.062	13.264 ± 0.030	−0.229	−0.360	−0.294	1.226 ± 0.079
42°C — 0′[d]	13.742 ± 0.040	14.104 ± 0.092	—	—	—	—
42°C — 15′[d]	14.011 ± 0.139	14.698 ± 0.001	0.226	0.424	0.325	0.798 ± 0.077
42°C — 30′[d]	14.175 ± 0.031	15.074 ± 0.004	0.557	0.517	0.537	0.689 ± 0.013
42°C — 60′[d]	15.030 ± 0.012	15.823 ± 0.038	0.396	0.467	0.432	0.741 ± 0.026
42°C — 180′[d]	13.664 ± 0.063	13.916 ± 0.119	−0.239	0.019	−0.110	1.079 ± 0.138

[a] Example of relative *AeaHsc70* ΔCt calculation using *Actin* as reference gene. Relative *AeaHsc70* $\Delta\Delta C_t$ at a time point = *AeaHsc70*ΔC_t–*AeaHsc70*ΔC_t-0 at 0 h point.
[b] First instar larvae.
[c] Pupae (<16 h).
[d] Seven day-old female.

treatment of *Ae. aegypti* larvae (21.28 ± 0.119), more than a 44-fold relative increase over that found in the 1 h 23°C control (0.458 ± 0.023) (Table 3, Fig. 2A). After 3 h at 42°C, *AeaHsp83* gene expression (8.296 ± 1.254) increased significantly (50-fold) when compared with the untreated control (0.156 ± 0.016) (Table 3, Fig. 2A, supplementary data Table 6).

The *AeaHsc70* expression level increased significantly but at a much lower level (fourfold) after 15 min at 42°C treatment (2.132 ± 0.959) in *Ae. aegypti* larvae compared with the control (0.536 ± 0.189) (Fig. 3A and Table 4, supplementary data Table 7A). A slight but insignificant increase in expression of *AeaHsc70* mRNA was found after heat-shock at 1 h 42°C treatment (1.370 ± 0.160) compared with the control (1.169 ± 0.050) (Fig. 3A, Table 4, supplementary data Table 7).

High Temperature Effects on Relative RNA Expression Levels of *AeaHsp26*, *AeaHsp83*, and *AeaHsc70* in *Ae. aegypti* Pupae. We examined *AeaHsp26*, *AeaHsp83*, and *AeaHsc70* expression over time after heat shock treatment in *Ae. aegypti* pupae (Tables 2–4). According to our qPCR data, expression of a small *AeaHsp26* was up-regulated after 15 min at 42°C treatment of *Ae. aegypti* pupae (350.52 ± 21.24) compared with the control (2.773 ± 0.183) (Table 2). At 1 h post 42°C treatment, *AeaHsp26* expression was significantly greater (929.46 ± 6.742) in *Ae. aegypti* pupae when compared with the control (6.288 ± 1.594) (Table 2). In general, relative *AeaHsp26* expression increased significantly during 42°C treatment (40- to 140-fold) over that found in the control (Fig. 1B, supplementary data Table 5).

The expression of *AeaHsp83* was also upregulated after heat shock treatment of *Ae. aegypti* pupae, ranging from 4- to 13-fold after 15 min and 1 h, respectively (Table 3 and Fig. 2B). After 3 h at 42°C, *AeaHsp83* expression (7.988 ± 0.532) was lower than that after 1 h treatment of *Ae. aegypti* pupae (19.369 ± 0.800) (Table 3, Fig. 2B).

The expression of *AeaHsc70* was upregulated fourfold after 1 h at 42°C in *Ae. aegypti* pupae (2.323 ± 0.076) over that of the control (0.622 ± 0.059) and continued to increase through 3 h at 42°C treatment (4.228 ± 0.232) (Table 4 and Fig. 2C). In addition, we also examined 7-d-old female *Ae. aegypti* after heat treatment. The expression of *AeaHsp26* was up-regulated more than a 200-fold after 30 min at 42°C treatment of female *Ae. aegypti* (65.352 ± 0.756), when compared with the controls (0.319 ± 0.005) (Table 2, Fig. 1C). The expression of *AeaHsp26* decreased after 1 h at 42°C (37.809 ± 0.554) and then increased after 3 h at 42°C (62.901 ± 0.382) (Table 2).

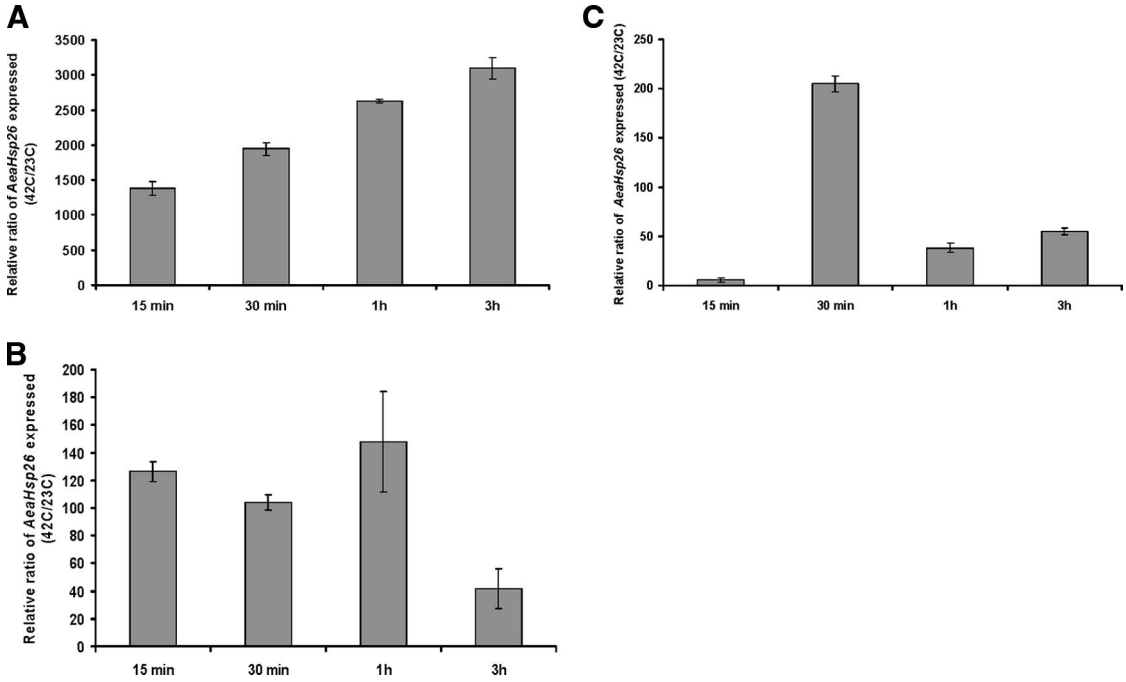

Fig. 1. qPCR results showing the relative ratio of *AeaHsp26* expressed (up regulated) after 42°C heat shock treatment compared with the 23°C control over time with SD for three replicates. (A) *AeaHsp26* genes differentially expressed after 42°C heat shock treatment of *Ae. aegypti* larvae compared with the 23°C control at 15, 30, 60, and 180 min. (B) *AeaHsp26* genes differentially expressed after 42°C heat shock treatment of *Ae. aegypti* pupae compared with the 23°C control at 15, 30, 60, and 180 min. (C) *AeaHsp26* genes differentially expressed after 42°C heat shock treatment of *Ae. aegypti* females compared with the 23°C control at 15, 30, 60, and 180 min.

The expression of *AeaHsp83* was slightly upregulated after 42°C treatment of adults, ranging from 2- to 12-fold compared with the control (23°C) (Table 3, Fig. 2C). However, the expression of *AeaHsc70* was not significantly different between the 42°C treatment and the control females (23°C) (Table 4, Fig. 3C).

Discussion

HSPs such as *Hsp90*, *Hsp70*, and *Hsp27* are induced in response to a variety of physiological environmental stresses including heat, reactive oxygen species, and anticancer drugs in human cells (Zhuang et al. 2010). Increased expression of small heat shock proteins (sHSPs) is known to be a key regulatory mechanism in extending tolerance to a variety of environmental stresses, such as the wasp *Venturia canescens* (Gravenhorst) after exposure to different temperatures (Reineke 2005); in *Anopheles* vectors due to interaction with *Plasmodium* parasites (Lefevre et al. 2007) and *Drosophila* in response to injuries and aging (Morrow et al. 2004). In addition to enhance stress resistance, HSPs are chaperones thought to increase lifespan and prevent apoptosis and neurodegenerative diseases. In *Drosophila*, neuronal expression of *Hsp26* or *Hsp27* increases lifespan and resistance to oxidative stress, although the function of neurola expression between *Hsp26* and *Hsp27* is different (Liao et al. 2008). Overexpression of either *Hsp26* or *Hsp27* increases stress resistance and extends the mean lifespan by 30% in transgenic *Drosophila* (Wang et al. 2004). Although suppressing expression of *Hsp23* and *Hsp70* in flies by using RNAi does not alter the decision to enter diapause or the duration of diapause in insects, it does have an effect on ability of pupae to survive low temperatures, and up-regulation of *Hsp23* and *Hsp70* during diapause is a major factor contributing to cold-hardiness of overwintering insects (Rinehart et al. 2007). In contrast, the *Sarcophaga crassipalpis* (Macquart) shows upregulation of *Hsp23* and *Hsp70* and downregulation of *Hsp90* during its pupal diapause (Tachibana et al. 2005). In *Drosophila triauraria*, *Hsp23*, *Hsp26*, and *Hsp83* are not regulated as a function of diapause and are not involved in the expression of diapause in this species (Goto and Kimura 2004). In nature, mosquitoes may be subjected to temperature extremes and other stressors and have developed mechanisms to survive these conditions. Our quantitative RT-PCR data revealed significant differences in the expression of *AeaHsp26* (sHSP 26 kDa) in response to heat shock treatment of *Ae. aegypti* larvae, pupae, and females. In the heat shock treatment of *Ae. aegypti* larvae, *AeaHsp26* expression levels were up-regulated >1,300 times compared with the control (Fig. 1A), and were much higher than in the heat shock treatment of pupae and adults. Elevated gene expression of *AeaHsp26* suggests that it may play a critical role in

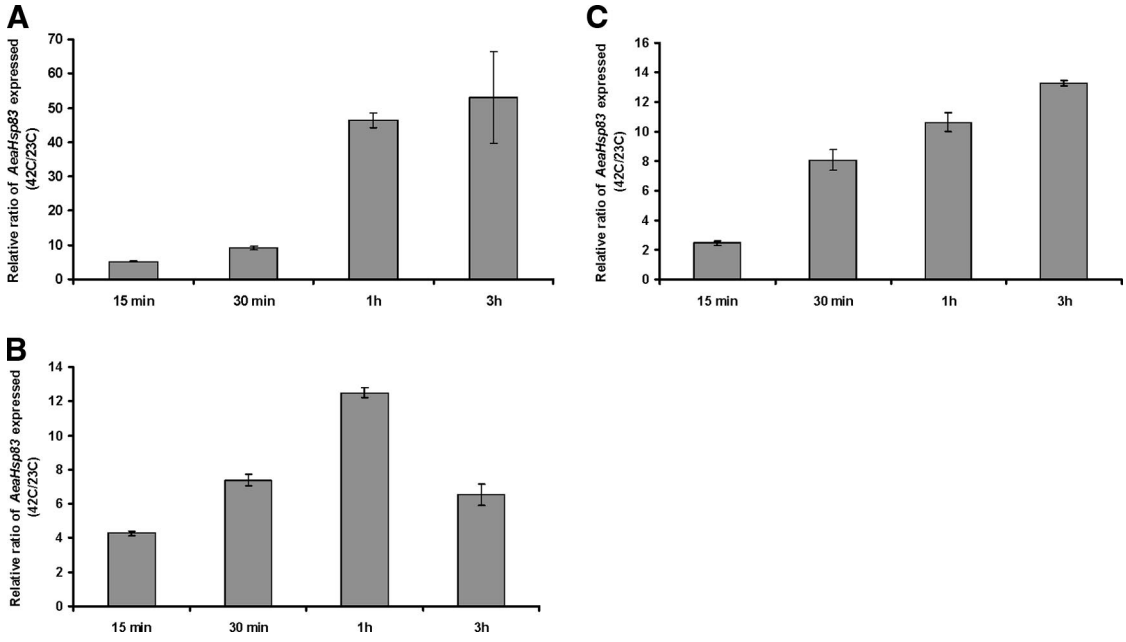

Fig. 2. qPCR results showing the relative ratio of *AeaHsp83* expressed (up regulated) after 42°C heat shock treatment compared with the 23°C control over time, with SD for three replicates. (A) *AeaHsp83* genes differentially expressed after 42°C heat shock treatment of *Ae. aegypti* larvae compared with the 23°C control at 15, 30, 60, and 180 min. (B) *AeaHsp83* genes differentially expressed after 42°C heat shock treatment of *Ae. aegypti* pupae compared with the 23°C control at 15, 30, 60, and 180 min. (C) *AeaHsp83* genes differentially expressed after 42°C heat shock treatment of *Ae. aegypti* females compared with the 23°C control at 15, 30, 60, and 180 min.

response of larval mosquitoes to high temperature conditions but less of a role in pupae and adults.

Hsp90, a highly conserved essential protein in all eukaryotes, is a molecular chaperone that is known to facilitate the conformational maturation of a diverse range of proteins involved in different signal transduction pathways during development (Barginear et al. 2008, Martins et al. 2009, Pisa et al. 2009, Tariq et al. 2009). In a previous study, *Hsp90* activity was shown to be required for efficient targeting of human argonaut proteins to processing bodies and stress granules and pharmacological inhibition of *Hsp90* is associated with reduced microRNA- and short interfering RNA-dependent gene silencing (Pare et al. 2009). Interestingly, *Hsp90* inhibitors promote proteasomal degradation of progrowth and prosurvival *Hsp90* client proteins and induce apoptosis of human lymphoma cells (Rao et al. 2009). *Hsp90* is important for maintaining *Sp1* (a human transcription factor involved in gene expression in the early development of an organism) stability during mitosis (Wang et al. 2009). *Hsp90* is overexpressed in poor-prognosis primary acute myeloid leukemia cells and plays a role in cell survival and resistance to chemotherapy (Flandrin et al. 2008). In a *Caenorhabditis elegans* (Maupas) Cedaf-21 (*Hsp90*) null mutant, the *C. elegans Hsp90* endogenous gene provided full rescue of the daf-21 mutant, while *Haemonchus contortus* (Cobb) *Hsp90* provided partial rescue that indicates this parasite gene can functionally complement in *C. elegans* (Gillan et al. 2009). In *Delia antiqua* (Meigen), *Hsp90* expression levels increase and decrease differently during development and physiology of summer- and winter-diapauses (Chen et al. 2005). In nondiapausing pupae of *D. antiqua*, *Hsp90* expression is up-regulated following cold- and heat-stresses (Chen et al. 2005). One study shows that *Hsp90* is the best candidate for an evolved mechanism that regulates the expression of genetic and phenotypic variability involved in thermotolerance in natural populations of *D. melanogaster* (Meigen) from eastern Australia (Sgro et al. 2008). Genetic approaches show that the *Hsp90* chaperone (encoded by *Hsp83*) is a localization factor for two mRNAs (nanos and pgc) and is essential for development of both the abdominal segments and primordial germ cells in the *Drosophila* embryo (Song et al. 2007). Other interesting studies show that *Hsp90* contributes to, does not control, the buffering of phenotypic variation in wing shape in *D. melanogaster* (Debat et al. 2006). It is also a specific signal transducer at the interface of several developmental pathways (Rutherford and Lindquist 1998). In the current study, the *Hsp90* protein encoded by the *AeaHsp83* gene is up-regulated in response to the heat shock treatment in *Ae. aegypti.* Since *Hsp90* protein is an important molecular chaperone and its function includes assisting in protein folding and cell signaling, elevated gene expression of *AeaHsp83* indicates that it is an important gene for surviving environmental stress induced by high temperatures.

Heat shock cognate 70 (*Hsc70*), which has 85% nucleotide identity with human *Hsp70,* and *Hsp70*

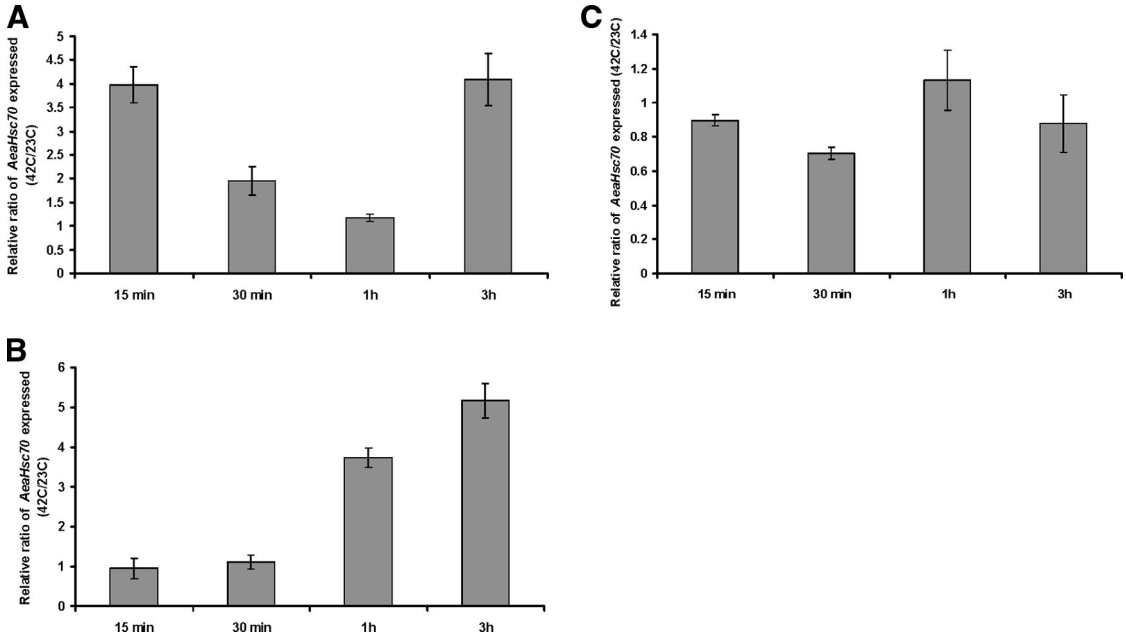

Fig. 3. qPCR results showing the relative ratio of *AeaHsc70* expressed (up regulated) after 42°C heat shock treatment compared with the 23°C control over time with SD for three replicates. (A) *AeaHsc70* genes differentially expressed after 42°C heat shock treatment of *Ae. aegypti* larvae compared with the 23°C control at 15, 30, 60, and 180 min. (B) *AeaHsc70* genes differentially expressed after 42°C heat shock treatment of *Ae. aegypti* pupae compared with the 23°C control at 15, 30, 60, and 180 min. (C) *AeaHsc70* genes differentially expressed for after 42°C heat shock treatment of *Ae. aegypti* females compared with the 23°C control at 15, 30, 60, and 180 min.

belong to the *Hsp70* family, but they may function differentially in the intracellular trafficking (Goldfarb et al. 2006). *Hsc70* is expressed in all developmental stages, from embryo to adult in *Chironomus* (Karouna-Renier et al. 2003). Unlike *Hsp70*, which is highly regulated in response to high temperature in *Ae. aegypti* (Gross et al. 2009), *Hsc70* is constitutively expressed in *Sesamia nonagrioides* (Lefebvre) (Gkouvitsas et al. 2009). High temperature stress during diapause has no further effect on transcript levels of *Hsc70* in *S. nonagrioides* and the *S. crassipalpis* (Rinehart et al. 2007, Gkouvitsas et al. 2009). In *Anopheles gambiae* (Giles), the expression of *Hsc70B* is induced by heat shock and arbovirus infection, and *Hsc70B* protein is expressed to cope with cellular stress imposed during infection (Kang et al. 2008). Using double-stranded RNA interference to investigate *Hsc70B*, it was determined that it has important roles in homeostasis and suppression of o'nyong-nyong virus replication in the vector, *An. gambiae* (Sim et al. 2007). The present data also showed that high temperature has little effect on the RNA expression of *AeaHsc70*, which indicates *AeaHsc70* gene expression may be regulated by environmental factors other than the temperature.

Environmental regulation of gene expression plays an important and often critical role in the development and physiological response of many organisms including mosquitoes. Here we have examined in detail the activity of the heat shock or stress proteins *AeaHsp26* and *AeaHsp83* in *Ae. aegypti* larvae and pupae and found that expression of these HSPs was highly regulated in response to heat stress. The current study has shown that *AeaHsp26* and *AeaHsp83* are important markers of stress and may function as critical proteins to protect and enhance survival of *Ae. aegypti* larvae and pupae.

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