Developmental and Environmental Regulation of *AaeIAP1* Transcript in *Aedes aegypti*

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ABSTRACT Apoptosis (programmed cell death) is a tightly regulated physiological process. The inhibitors of apoptosis proteins (IAPs) are key regulators for apoptosis. An inhibitor of apoptosis protein gene *IAP1* was recently cloned from *Aedes aegypti* (L.) (*AaeIAP1*, GenBank accession no. DQ993355); however, it is not clear whether *AaeIAP1* is developmentally and environmentally regulated. In this study, we applied quantitative polymerase chain reaction (PCR) to investigate the expression levels of the *AaeIAP1* transcript in different developmental stages and under different environmental conditions. Our results revealed that the expression of the *AaeIAP1* transcript was detectable in all life stages of *Ae. aegypti*, with significantly higher levels in pupal and adult stages than in larval stages. Furthermore, when *Ae. aegypti* was exposed to all stressful environmental conditions (e.g., low and high temperatures, UV radiation, acetone, and permethrin insecticide treatment), the expression level of *AaeIAP1* transcript was increased significantly. Our results suggest that *AaeIAP1* might play an important role in both the physiological development of *Ae. aegypti* and stress-induced apoptosis.

KEY WORDS inhibitor of apoptosis, inhibitor of apoptosis protein, *Aedes aegypti*, quantitative PCR, developmental regulation

Apoptosis is an evolutionarily conserved event in the development of multicellular organisms to remove unwanted, damaged, mutated, or infected cells (Liu and Hengartner 1999, Twomey and McCarthy 2005). Inhibitors of apoptosis proteins (IAPs) are a family of potent antiapoptotic proteins that were originally discovered in insect baculoviruses (*Cydia pomonella* granulosis virus and *Orgyia pseudotsugata* nuclear polyhedrosis virus) (Crook et al. 1993, Birnbaum et al. 1994, Clem and Miller 1994), and they have since been identified in various other viruses (Chacon et al. 1995, Delhon et al. 2006), yeast (Walter et al. 2006), invertebrates (Hay et al. 1995, Huang et al. 2001, Muro et al. 2002), and vertebrates (Digby et al. 1996, Liston et al. 1996, Ambrosini et al. 1997, Vitte-Mony et al. 1997, Wagenknecht et al. 1999). Many IAPs are capable of blocking apoptosis when they are overexpressed in cells of other species (Beidler et al. 1995, Hawkins et al. 1996, Hawkins et al. 1998, Li et al. 2007), suggesting that IAPs target a conserved step in the apoptosis pathway.

In insects, *Drosophila* is the leading insect model for the study of apoptosis regulation. To date, four *Drosophila* IAPs (DIAP1, DIAP2, Deterin, and Bruce) have been reported (Hay et al. 1995, Jones et al. 2000, Qiu et al. 2004). However, only limited information is available on apoptotic regulation in mosquitoes, despite the central role that these vectors play in disease transmission. Here, we present the developmental regulation of an IAP1 homolog from *Aedes aegypti* (L.), *AaeIAP1*, the vector of yellow fever and dengue viruses, both of which can cause severe human morbidity and mortality (Patterson 1992, Huhtamo et al. 2006). Furthermore, we report that the expression level of *AaeIAP1* transcript is induced to significantly ($P < 0.001$) higher levels in *Ae. aegypti* under stressful environmental conditions (e.g., UV radiation, heat-shock, and pesticide treatment). This is the first report of the developmental and environmental regulation of an IAP homolog in *Ae. aegypti*.

Materials and Methods

Sampling for Different Developmental Stages of *Ae. aegypti*. The Orlando strain of *Ae. aegypti* was reared in the insectary of the Mosquito and Fly Research Unit at Center for Medical, Agricultural, and Veterinary Entomology, ARS–USDA in Gainesville, FL. Mosquitoes were reared using methods described

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previously (Pridgeon et al. 2007). Briefly, eggs were hatched in a flask, and the larvae were held overnight in the flask and then transferred to a plastic tray containing distilled water. Larval diet was added to each tray. Mosquitoes were reared in an environmental chamber set with a temperature ranging from 26 to 28°C and 80% RH. Incandescent lighting was set to a crepuscular profile with a photoperiod of 14:10 (L:D) h, including 2 h of simulated dawn and dusk. Larval samples were collected at different times after hatching. At each time, six voucher samples were collected, with each voucher containing 20–30 mosquito larvae. Three vouchers were quickly frozen and stored at −80°C for later RNA isolation, and the remainder of the vouchers were used to measure the transverse diameter of the head capsule, which has been a reliable method for identification of larval instars according to Christophers (1960). The transverse diameter of the head capsule was measured under a microscope (model Stemi SV8, Carl Zeiss, Thornwood, NY) connected to a camera (model 11.2 Color Mosaic, Diagnostic Instruments, Sterling Heights, MI). Pupal samples were collected at different times to get early stage pupae, middle stage pupae, and late stage pupae samples and stored at −80°C for later RNA isolation. Adults were held in a screened cage and provided 10% sucrose ad libitum. Emerged adults were transferred to a new screened cage, and adult samples were collected at different times after emergence. Bovine blood in 1% heparin placed in a pig intestine warmed to 37°C was provided to adults twice a week. Freshly laid eggs were collected, and eggs were obtained at different times (1, 3, and 6 d old) and stored at −80°C.

Mosquito Treatments with UV Radiation, High or Low Temperature, or Pesticide Permethrin. For UV treatment, thirty 7-d-old females sorted into 4-oz plastic cups and covered by cotton mesh were supplied with 10% sucrose and placed on a UV transilluminator (Fisher, Hampton, NH) for UV radiation at 312 nm. Samples were collected at different times (15 min, 30 min, and 2 h postirradiation). For temperature treatments, thirty 7-d-old females sorted into 4-ounce plastic cups and supplied with 10% sucrose were placed in incubators at 4, 42, or 55°C. Samples were collected at different times. For the treatment with permethrin, this pesticide was diluted in acetone and topically applied at a sublethal dose to individual mosquitoes. Before permethrin treatment, thirty 7-d-old females were briefly anesthetized for 30 s with carbon dioxide and placed on a 4°C chill table (BioQuip Products, Rancho Dominguez, CA). A droplet of 0.5 μl of permethrin solution at sublethal dose (2.5×10^{-5} μg) was applied to the dorsal thorax of the mosquito by using a 700 series syringe and a PB 600 repeating dispenser (Hamilton, Reno, NV). Samples treated with permethrin were collected at 0.5, 3, 6, 12, and 24 h after application. Control samples with 0.5 μl of acetone treatment were also collected at the same times as those receiving the permethrin treatment. After treatment, mosquitoes were kept in plastic cups and supplied with a 10% sucrose solution. Temperature and relative humidity were maintained at 26°C and 80%, respectively. All treatments were replicated three times and samples were stored at −80°C for later RNA isolation.

***Ae. aegypti* RNA Extraction and cDNA Synthesis.** Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Briefly, 0.8 ml of TRIzol reagent was added to each sample (80 mg). Samples were homogenized using a Tissuemiser (Fisher), and then they were incubated at room temperature for 5 min. Chloroform (0.2 ml) was added to each sample, and the mixture was vortexed for 1 min and kept at room temperature for 3 min. After centrifugation at 12,000 × *g* for 15 min at 4°C, the clear supernatant (containing the RNA) was transferred to 1.5-ml microcentrifuge tubes. RNA precipitation was carried out by adding 0.5 ml of isopropanol to each sample followed by incubation at room temperature for 30 min and centrifugation at 12,000 × *g* for 15 min. The RNA pellets were washed with 1 ml of 75% ethanol in diethyl pyrocarbonate (DEPC)-treated water, followed by centrifugation at 12,000 × *g* for 10 min. The pellets were dried and suspended in an appropriate volume of DEPC-water according to the size of the pellet (≈10–50 μl). Total RNA was then treated with amplification grade DNaseI (Invitrogen). RNA ethanol precipitation was carried out after adding 1/10 volume of 5 M NaCl to the DNaseI digested RNA solution. The RNA pellets were then washed with 1 ml of 75% ethanol in DEPC-treated water, followed by centrifugation at 12,000 × *g* for 10 min. The pellets were dried and suspended in an appropriate volume of DEPC water according to the size of the pellet (≈10–50 μl). A 1.0% denaturing agarose/formaldehyde gel was used to test the integrity and quality of the total RNA. The RNA concentration was assessed by the absorbance of RNA at 260 nm in a Genesys 10 UV spectrophotometer (Thermo Scientific, Hampton, NH). For first-stand cDNA synthesis, a 20-μl reaction mixture containing total RNA (3 μg), oligo$(dT)_{20}$ primer (50 μM) (Invitrogen), and 10 mM dNTP mix was incubated at 70°C for 10 min and then placed on ice. After briefly spinning the reaction mixture, 5X cDNA synthesis buffer, 0.1 M dithiothreitol, RNase inhibitor (40 U/μl), and Cloned avian myeloblastosis virus reverse transcriptase (15 U/μl) (Invitrogen) were added to the reaction mixture. The mixture was then incubated at 42°C for 58 min followed by 95°C for 15 min in a 9600 thermocycler (Applied Biosystems, Foster City, CA).

Quantitative Polymerase Chain Reaction (PCR). Treated *Ae. aegypti* were frozen at −80°C for subsequent RNA extraction. Total RNA was prepared and reverse transcribed as described above. Quantitative PCR was performed with SYBR Green PCR Master Mix on an ABI 7300 quantitative PCR System (Applied Biosystems, Foster City, CA). Primers for the amplification of the *AaeIAP1* gene were the forward primer IAP-911 F (5′-CCTCAAAGACTGGGAAGCTG-3′) and the reverse primer IAP-1133R (5′-TGACTGAAGCGAGGATGTTG-3′). Primers for the amplification of the actin gene were forward primer set Actin-152 F (5′-AGGACTCGTACGTCGGTGAC-3′) and Actin-590R (5′-CGTTCAGTCAGGATCTTC-3′).

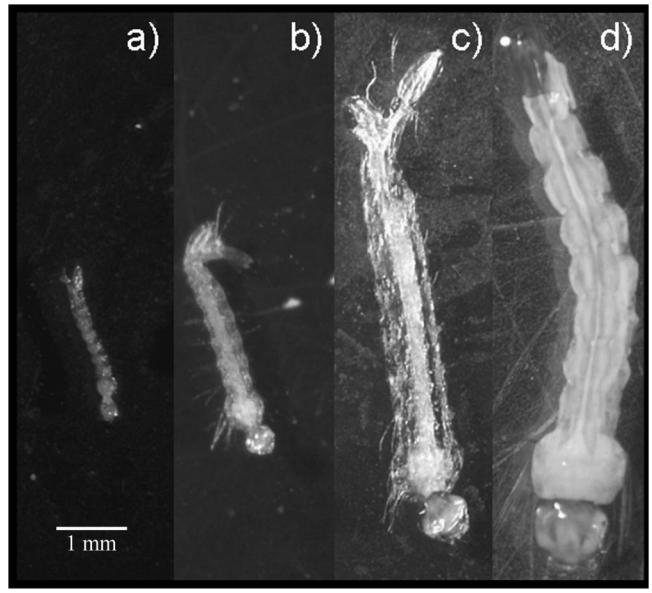

Fig. 1. Representative picture showing the head capsule of instars I–IV. (a) Instar I with the transverse diameter of the head capsule of 0.25 mm. (b) Instar II with the transverse diameter of the head capsule of 0.45 mm. (c) instar III with the transverse diameter of the head capsule of 0.71 mm. (d) Instar IV with the transverse diameter of the head capsule of 1.00 mm.

The relative expression level of *AaeIAP1* was normalized to actin level and calculated using the equation $100 \times 2^{-\Delta CT}$, where $\Delta C_t = C_t(AaeIAP1) - C_t$ (actin) (Portereiko et al. 2006). Data were analyzed by analysis of variance (ANOVA) by using SigmaStat statistical analysis software (SAS Institute, Cary, NC) where the differences in the mean values were analyzed statistically.

Table 1. Transverse diameters of larvae head capsules

Sample stage	Sample name	Sample time	Transverse diameters of the head capsules (mm)										Mean ± SD
			1	2	3	4	5	6	7	8	9	10	
First instar	Larvae 1	6 h ph[a]	ND[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Larvae 2	9 h ph	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Larvae 3	23 h ph	0.30	0.34	0.25	0.28	0.32	0.28	0.31	0.29	0.32	0.27	0.296 ± 0.027
	Larvae 4	30 h ph	0.25	0.27	0.32	0.26	0.26	0.28	0.31	0.30	0.29	0.28	0.282 ± 0.023
	Larvae 5	33 h ph	0.32	0.25	0.29	0.24	0.31	0.29	0.27	0.26	0.26	0.32	0.281 ± 0.029
	Larvae 6	36 h ph	0.29	0.31	0.30	0.29	0.28	0.32	0.24	0.22	0.23	0.27	0.275 ± 0.034
	Larvae 7	39 h ph	0.20	0.30	0.31	0.30	0.27	0.24	0.25	0.26	0.28	0.30	0.271 ± 0.035
Second instar	Larvae 8	48 h ph	0.49	0.51	0.49	0.45	0.52	0.43	0.46	0.42	0.49	0.47	0.476 ± 0.033
	Larvae 9	51 h ph	0.42	0.48	0.44	0.42	0.45	0.45	0.50	0.41	0.47	0.52	0.456 ± 0.036
	Larvae 10	54 h ph	0.49	0.47	0.42	0.44	0.46	0.48	0.52	0.45	0.51	0.45	0.469 ± 0.031
	Larvae 11	57 h ph	0.50	0.49	0.47	0.45	0.45	0.44	0.49	0.38	0.39	0.42	0.448 ± 0.042
	Larvae 12	60 h ph	0.42	0.44	0.44	0.42	0.50	0.43	0.49	0.46	0.48	0.45	0.453 ± 0.029
	Larvae 13	63 h ph	0.44	0.49	*0.75*	0.43	*0.70*	0.49	0.44	0.47	0.44	0.60	0.475 ± 0.056
Third instar	Larvae 14	72 h ph	0.80	*0.49*	0.68	0.76	0.80	0.78	0.78	0.76	0.80	0.77	0.770 ± 0.037
	Larvae 15	75 h ph	0.70	*0.47*	*0.46*	0.74	0.75	0.71	0.72	0.77	0.74	0.71	0.730 ± 0.024
	Larvae 16	78 h ph	0.76	0.78	0.74	0.71	0.72	0.77	0.73	0.80	0.70	0.77	0.748 ± 0.033
	Larvae 17	81 h ph	0.78	0.73	0.68	0.72	0.73	0.74	0.72	0.71	0.69	0.62	0.712 ± 0.042
	Larvae 18	84 h ph	*0.93*	0.66	0.73	0.71	0.69	0.68	0.61	0.76	0.71	0.60	0.683 ± 0.053
	Larvae 19	87 h ph	0.74	0.69	0.71	0.77	0.73	0.87	*0.93*	0.67	0.72	*0.96*	0.737 ± 0.062
Fourth instar	Larvae 20	99 h ph	*0.69*	0.88	1.03	0.91	0.89	1.08	1.02	0.93	0.98	0.93	0.961 ± 0.070
	Larvae 21	105 h ph	0.97	0.98	0.93	1.04	1.02	1.06	0.99	1.11	0.88	0.89	0.987 ± 0.074
	Larvae 22	111 h ph	0.96	1.01	0.94	0.94	0.90	0.97	0.81	0.97	0.93	1.06	0.949 ± 0.066
	Larvae 23	129 h ph	0.96	0.96	0.92	1.00	0.96	1.02	1.07	0.98	0.87	0.92	0.977 ± 0.048
	Larvae 24	132 h ph	0.93	0.98	0.95	0.97	0.91	0.96	0.97	0.92	0.93	0.93	0.945 ± 0.024
	Larvae 25	145 h ph	0.98	0.97	0.99	0.99	1.04	1.06	1.05	0.06	1.00	0.96	0.910 ± 0.301
	Larvae 26	148 h ph	1.01	1.08	1.09	1.01	1.07	1.03	1.00	1.12	1.11	0.99	1.051 ± 0.048

Numbers in italics are not included in the statistic analysis.
[a] Posthatch.
[b] Not determined.

Table 2. Expression of *AaeIAP1* at different developmental stages

Sample stage	Sample name	Sample time	Cycle threshold (Ct) ± SD		Relative *AaeIAP1* expression level			
			Actin	AaeIAP1	ΔCt-1	ΔCt-2	ΔCt-3	$100 \times 2^{-\Delta Ct}$ ± SD
Egg	Egg 1	1 d	19.520 ± 0.073	23.705 ± 0.038	4.185	3.480	3.440	7.891 ± 2.077
	Egg 2	3 d	19.345 ± 0.055	21.970 ± 0.001	2.625	2.970	2.440	15.801 ± 2.855
	Egg 3	6 d	22.215 ± 0.121	24.095 ± 0.021	1.880	1.980	2.000	25.839 ± 1.164
First instar	Larvae 1	6 h[a]	17.415 ± 0.022	21.175 ± 0.023	3.760	3.730	3.610	7.703 ± 0.431
	Larvae 2	9 h	17.405 ± 0.009	20.765 ± 0.050	3.360	3.791	3.018	9.769 ± 2.560
	Larvae 3	23 h	21.250 ± 0.000	23.435 ± 0.060	2.185	2.246	2.200	21.613 ± 0.473
	Larvae 4	30 h	16.825 ± 0.018	22.280 ± 0.028	5.455	6.110	5.832	1.828 ± 0.421
	Larvae 5	33 h	16.245 ± 0.047	20.995 ± 0.017	4.750	5.160	5.210	3.072 ± 0.560
	Larvae 6	36 h	16.065 ± 0.033	21.265 ± 0.162	5.200	5.250	5.150	2.721 ± 0.094
	Larvae 7	39 h	16.895 ± 0.019	21.525 ± 0.162	4.630	4.846	4.600	3.880 ± 0.351
Second instar	Larvae 8	48 h	19.580 ± 0.026	22.185 ± 0.006	2.605	2.449	2.629	16.975 ± 1.173
	Larvae 9	51 h	16.980 ± 0.028	22.130 ± 0.022	5.150	5.454	5.190	2.621 ± 0.289
	Larvae 10	54 h	18.150 ± 0.026	21.390 ± 0.014	3.240	3.071	3.300	10.879 ± 0.910
	Larvae 11	57 h	21.245 ± 0.023	24.130 ± 0.039	2.885	3.030	3.130	12.401 ± 1.066
	Larvae 12	60 h	17.570 ± 0.026	22.355 ± 0.063	4.785	5.150	5.050	3.154 ± 0.422
	Larvae 13	63 h	17.970 ± 0.015	22.735 ± 0.049	4.765	5.830	5.430	2.585 ± 0.987
Third instar	Larvae 14	72 h	16.730 ± 0.003	22.205 ± 0.007	5.475	5.970	5.927	1.829 ± 0.364
	Larvae 15	75 h	17.670 ± 0.076	22.805 ± 0.020	5.135	6.542	5.442	2.073 ± 0.908
	Larvae 16	78 h	17.905 ± 0.019	22.570 ± 0.003	4.665	6.590	5.040	2.673 ± 1.486
	Larvae 17	81 h	18.005 ± 0.031	22.990 ± 0.046	4.985	4.437	5.141	3.536 ± 0.949
	Larvae 18	84 h	17.350 ± 0.041	22.345 ± 0.205	4.995	5.439	5.340	2.637 ± 0.440
	Larvae 19	87 h	17.460 ± 0.020	23.065 ± 0.207	5.605	5.578	5.540	2.099 ± 0.047
Fourth instar	Larvae 20	99 h	18.235 ± 0.015	22.140 ± 0.045	3.905	3.904	4.145	6.336 ± 0.592
	Larvae 21	105 h	19.125 ± 0.040	22.510 ± 0.179	3.385	3.081	3.140	10.912 ± 1.185
	Larvae 22	111 h	19.110 ± 0.073	22.110 ± 0.030	3.000	2.775	2.720	14.096 ± 1.411
	Larvae 23	129 h	20.380 ± 0.041	23.000 ± 0.018	2.620	2.775	2.652	15.598 ± 0.873
	Larvae 24	132 h	19.430 ± 0.040	22.845 ± 0.059	3.415	3.440	3.650	8.852 ± 0.771
	Larvae 25	145 h	19.540 ± 0.003	22.885 ± 0.389	3.345	3.975	3.726	7.919 ± 1.769
	Larvae 26	148 h	19.855 ± 0.027	22.815 ± 0.003	2.960	2.602	2.544	15.491 ± 2.311
Pupa	P1	154 h	18.230 ± 0.028	20.125 ± 0.004	1.895	1.825	1.623	29.196 ± 2.916
	P2	157 h	19.100 ± 0.031	20.565 ± 0.086	1.465	1.600	1.273	36.860 ± 4.226
	P3	169 h	19.105 ± 0.035	20.440 ± 0.039	1.335	1.473	1.090	40.881 ± 5.586
Adult	A1	1 d	19.950 ± 0.294	20.290 ± 0.014	0.340	0.540	0.140	79.511 ± 10.996
	A2	6 d	19.015 ± 0.004	20.705 ± 0.120	1.690	1.735	1.780	30.051 ± 0.938
	A3	12 d	18.605 ± 0.007	20.005 ± 0.049	1.400	1.170	1.370	40.341 ± 3.574

[a] Hours posthatch.

Results

To identify the instars of the larval samples, the transverse diameters of the head capsules were measured (Fig. 1). According to Christophers (1960), the most dependable method for identification of larvae is by measuring the transverse diameter of the head capsule. The successive larval instars I–IV of the head diameter should be ≈0.3, 0.45, 0.65, and 0.95 mm, respectively. Using the criterion of ≈0.3 mm for instar I, seven time point samples (L1–L7) up to 39 h posthatch were identified to be first instar, with mean head capsule transverse diameter ranging from 0.27 to 0.296 mm (Table 1). Similarly, using the criterion of ≈0.45 mm for instar II, six time points (L8–L13) ranging from 48 to 63 h posthatch were identified as second instars, with mean head capsule transverse diameters ranging from 0.448 to 0.476 mm (Table 1). Using the criterion of ≈0.65 mm for instar III, six time point samples (L14–L19) ranging from 72 to 87 h posthatch were identified as third instars, with mean head capsule transverse diameters ranging from 0.683 to 0.770 mm (Table 1). Using the criterion of ≈0.95 mm for instar IV, seven time point samples (L20–L26) ranging from 99 to 148 h posthatch were identified as fourth instars, with mean head capsule transverse diameters ranging from 0.945 to 1.051 mm (Table 1).

To understand the developmental regulation of *AaeIAP1* transcript under different physiological conditions, quantitative PCR was performed using egg, larval, pupal, and adult samples. As shown in Table 2 and Fig. 2, the expression of *AaeIAP1* transcript was detectable in all four developmental stages. Our results revealed that, although the expression of *AaeIAP1* transcript was detectable in all life stages of *Ae. aegypti*, the expression levels of *AaeIAP1* at each individual life stage were different. For example, in the embryonic stage, *AaeIAP1* was expressed significantly ($P < 0.001$) higher in the latter period (6 d old) than in the earlier period (1 d old) or the middle (3 d old) (Table 2; Fig. 2). Although *AaeIAP1* was detectable in all larval stages, its expression level in the fourth instar was significantly ($P < 0.001$) higher than that in the third instar. Our results also revealed that the expression level of *AaeIAP1* transcript in pupal and adult stages were significantly ($P < 0.001$) higher than that in the larval instars (Table 2; Fig. 2).

To understand whether extreme low or high temperatures affect the expression of *AaeIAP1*, we performed quantitative PCR to compare the expression levels of *AaeIAP1* transcript in different samples. As shown in Table 3 and Fig. 3, the expression levels of *AaeIAP1* transcript were increased significantly ($P <$

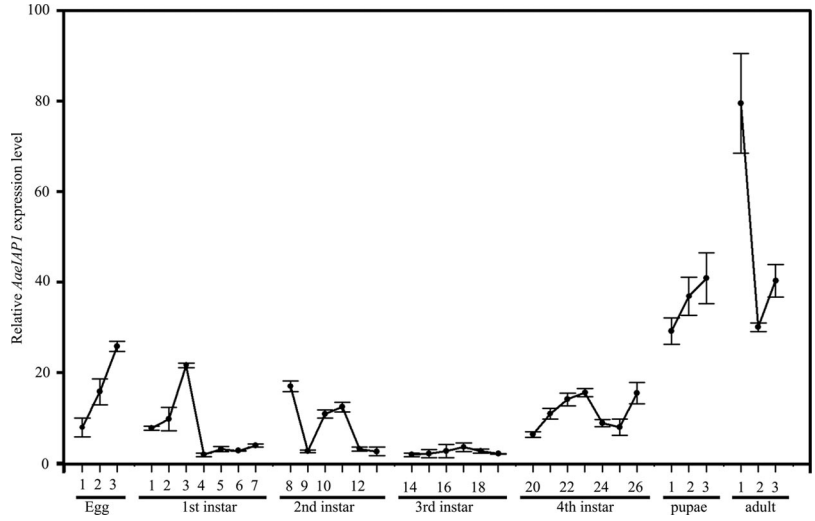

Fig. 2. Relative expression levels of *AaeIAP1* transcript in egg, larval, pupal, and adult stages detected by quantitative PCR. The ages of instar I (sample 1–7) are 6, 9, 23, 30, 33, 36, and 39 h posthatch, respectively. The ages of instar II (sample 8–13) are 48, 51, 54, 57, 60, and 63 h posthatch, respectively. The ages of instar III (sample 14–19) are 72, 75, 78, 81, 84, and 87 h posthatch, respectively. The ages of instar IV (sample 20–26) are 99, 105, 111, 129, 132, 145, and 148 h posthatch, respectively. Three samples of egg, pupal, and adult stages are representative samples of early, middle, and late stages.

0.001) upon the treatment of low (4°C) and high (42°C) temperatures at 3 h posttreatment. When we set the *AaeIAP1* level in the untreated mosquito as standard = 1, the treatment of low (4°C) and high (42°C) temperatures for 3 h increased the expression of *AaeIAP1* by ≈two-fold. The highest increase of *AaeIAP1* expression level was observed in mosquitoes treated at a temperature of 55°C for 20 min (Table 3; Fig. 3), which increased the expression level of *AaeIAP1* transcript by ≈18-fold.

To understand whether UV light exposure will affect the expression of *AaeIAP1*, we performed quantitative PCR after exposing adult mosquitoes to UV radiation. Our results revealed that UV radiation also significantly ($P < 0.001$) induced the expression level of *AaeIAP1* transcript (Table 3; Fig. 4). Thirty minutes after exposure to UV radiation, the expression level of *AaeIAP1* transcript was increased approximately eight-fold compared with the untreated control.

Table 3. Expression of *AaeIAP1* under different stress conditions

Sample name	Cycle threshold (Ct) ± SD		Relative *AaeIAP1* expression level			
	Actin	AaeIAP1	ΔCt-1	ΔCt-2	ΔCt-3	$100 \times 2^{-\Delta Ct} \pm$ SD
Untreated	19.065 ± 0.018	21.050 ± 0.026	1.985	2.020	1.300	30.177 ± 9.043
4°C-0.5 h	19.040 ± 0.395	19.505 ± 0.465	0.465	1.040	1.340	53.527 ± 17.009
4°C-3 h	20.695 ± 0.063	20.900 ± 0.563	0.205	0.830	0.560	70.279 ± 15.397
4°C-6 h	18.130 ± 0.009	19.885 ± 0.052	1.755	1.590	1.620	31.793 ± 1.906
4°C-12 h	16.980 ± 0.012	19.375 ± 0.120	2.395	2.470	2.350	18.892 ± 0.790
4°C-24 h	17.965 ± 0.063	20.535 ± 0.009	2.530	2.120	2.220	20.594 ± 2.943
42°C-0.5 h	17.720 ± 0.011	20.315 ± 0.067	2.650	2.340	2.540	17.626 ± 1.946
42°C-3 h	20.705 ± 0.118	21.745 ± 0.080	1.180	0.900	0.140	62.825 ± 24.643
42°C-6 h	17.855 ± 0.064	19.960 ± 0.019	2.105	2.020	2.070	23.906 ± 0.710
42°C-12 h	18.695 ± 0.051	20.600 ± 0.043	1.905	1.980	1.960	25.918 ± 0.701
42°C-24 h	30.905 ± 0.101	31.735 ± 0.931	0.830	0.230	0.550	69.939 ± 14.574
55°C-20 min	36.220 ± 0.058	33.820 ± 0.329	−2.40	−2.43	−2.45	535.197 ± 6.403
UV-15 min	26.920 ± 0.137	27.695 ± 0.024	0.775	0.660	0.860	58.941 ± 4.119
UV-30 min	35.655 ± 0.135	34.440 ± 0.009	−1.215	−1.130	−1.430	240.149 ± 26.227
UV-120 min	28.370 ± 0.510	27.040 ± 0.007	−1.330	−0.980	−1.280	230.496 ± 29.112
Acetone-0.5 h	17.925 ± 0.022	19.960 ± 0.126	2.035	2.020	1.930	25.100 ± 0.998
Acetone-3 h	20.580 ± 0.003	22.420 ± 0.024	1.840	2.210	2.220	23.670 ± 3.692
Acetone-6 h	21.215 ± 0.008	22.250 ± 0.037	1.035	1.190	1.070	46.755 ± 2.599
Acetone-12 h	27.290 ± 0.228	28.005 ± 0.006	0.715	1.020	0.970	53.761 ± 6.261
Acetone-24 h	19.480 ± 0.071	21.130 ± 0.056	1.650	1.750	1.740	30.510 ± 1.177
Permethrin-0.5 h	25.085 ± 0.009	26.370 ± 0.083	1.285	1.310	1.230	41.334 ± 1.178
Permethrin-3 h	33.025 ± 0.578	32.170 ± 0.590	−0.855	−0.810	−1.340	203.116 ± 43.421
Permethrin-6 h	33.280 ± 0.099	27.830 ± 0.022	−5.45	−5.86	−5.21	5860.752 ± 1516
Permethrin-12 h	18.860 ± 0.011	20.355 ± 0.388	1.495	1.030	1.760	37.991 ± 9.964
Permethrin-24 h	26.535 ± 0.011	27.400 ± 0.196	0.865	0.720	0.940	55.913 ± 4.381

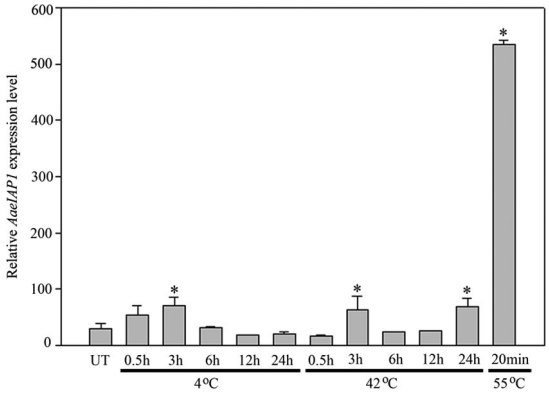

Fig. 3. Relative expression levels of *AaeIAP1* transcript in response to different temperature treatments. Data represent mean ± SD from three replicates. The asterisk (*) indicates there was a significant difference between the treatment and the untreated control.

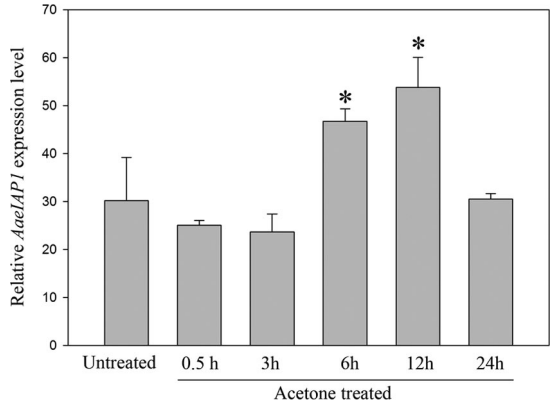

Fig. 5. Relative expression levels of AaeIAP1 transcript in response to acetone treatment. Data represent mean ± SD from three replicates. The asterisk (*) indicates there was a significant difference between the treatment and the untreated control.

Next, we examined the effect of acetone treatment on the expression of *AaeIAP1.* As shown in Table 3 and Fig. 5, acetone treatment did not affect the expression of *AaeIAP1* at 0.5 and 3 h posttreatment. However, at 6 and 12 h posttreatment, acetone significantly ($P < 0.001$) increased the expression of *AaeIAP1*, with increased level of *AaeIAP1* by approximately two-fold (Table 3; Fig. 5). At 24 h posttreatment with acetone, the expression levels of *AaeIAP1* were not significantly ($P > 0.05$) different between the untreated and the acetone-treated samples.

To determine the effect of permethrin on the expression level of *AaeIAP1* transcript, we topically applied permethrin to adult mosquitoes and performed quantitative PCR. Our results revealed that, at 0.5 h postpermethrin treatment, *AaeIAP1* expression levels were not significantly different ($P > 0.05$) between untreated and permethrin-treated mosquitoes (Table 3; Fig. 6). However, at 3 h postpermethrin treatment, the expression levels of *AaeIAP1* transcript were increased significantly ($P < 0.05$). The highest increase of *AaeIAP1* expression level was observed at 6 h postpermethrin treatment (≈200-fold greater than in the untreated control) (Table 3; Fig. 6).

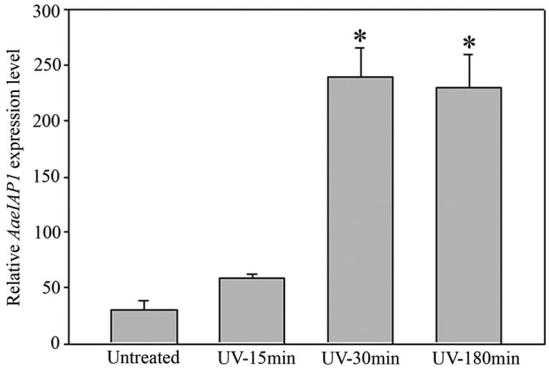

Fig. 4. Relative expression levels of AaeIAP1 transcript in response to UV irradiation. Data represent mean ± SD from three replicates. The asterisk (*) indicates there was a significant difference between the treatment and the untreated control.

Discussion

To identify the instars, the transverse diameters of the head capsule were measured. According to Christophers (1960), the successive instars I–IV of the head diameter should be ≈0.3, 0.45, 0.65, and 0.95 mm, respectively. Our results revealed that the mean head diameters of instars I–IV were ≈0.28, 0.46, 0.72, and 0.98 mm, respectively, which was only slightly different from the criteria set by Christophers (1960). We also noticed a difference in the duration of each instar. For example, our first instar lasted up to 39 h posthatch, whereas the first instar of *Ae. aegypi* described by Christophers (1960) lasted 28 h. The difference could be due to different rearing conditions, such as the water temperature and availability of food.

In the current study, we demonstrated the expression of *AaeIAP1* in different developmental stages of *Ae. aegypti* by quantitative PCR. Our results revealed that *AaeIAP1* was expressed throughout the developmental stages of *Ae. aegypti*, which is consistent with previous report on an IAP1 homolog expressed in all life stages of *Aedes triseriatus* (Say) and *Ae. aegypti* (Blitvich et al. 2002, Beck et al. 2007). However, the expression level of *AaeIAP1* in late embryonic stage was significantly higher than that in the early and middle embryonic stages. This observation is of great importance in that it suggests that *AaeIAP1* might play a functional role in embryonic development in *Ae. aegypti.* Our results also revealed that *AaeIAP1* expression levels in pupal and adult stages were significantly higher than that in the larval stage. Furthermore, at different times within each developmental stage, the expression levels of *AaeIAP1* were different, suggesting that *AaeIAP1* plays a pivotal role throughout the

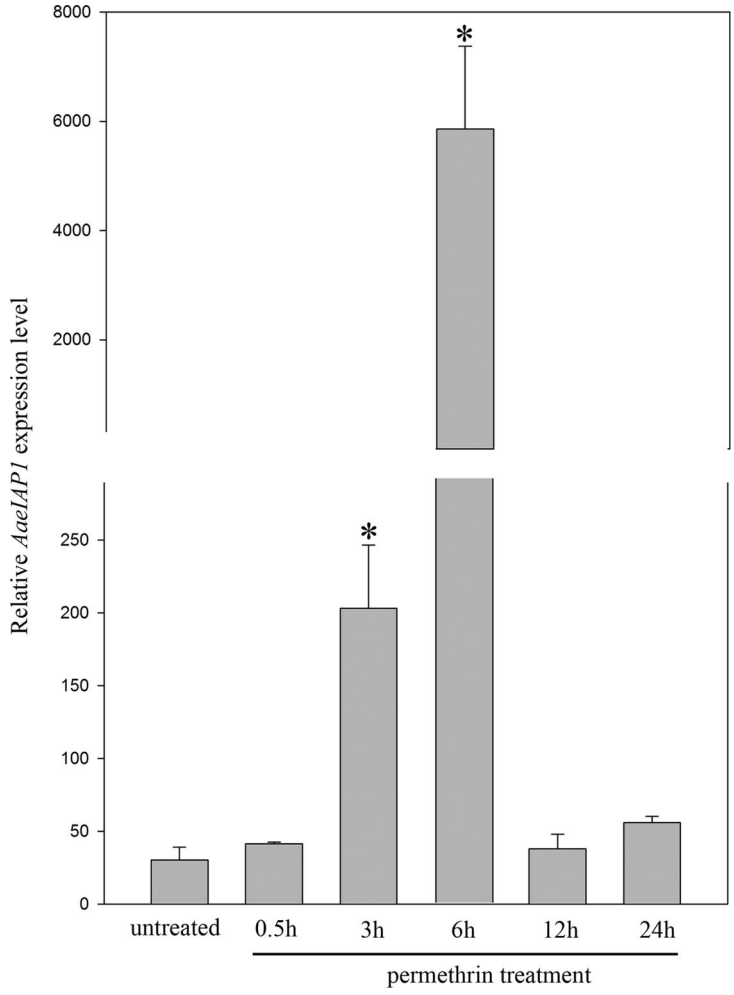

Fig. 6. Relative expression levels of AaeIAP1 transcript in response to permethrin treatment. Data represent mean ± SD from three replicates. The asterisk (*) indicates there was a significant difference between the treatment and the untreated control.

physiological process of the development of *Ae. aegypti*.

The transcriptional regulation of gene expression is a primary means by which insects adapt to a changing environment (Liu et al. 2007). Gene regulation in response to extreme temperatures has been extensively studied in insects. For example, in response to cold shock and heat shock, a 23-kDa small heat shock protein transcript was expressed much higher in nondiapausing flesh fly *Sarcophaga crassipalpis* Macquart (Yocum et al. 1998). However, gene regulation studies in response to cold or heat shock have been mainly focused on heat shock protein transcript. Knowledge of response of other genes to cold and heat shock is very limited. In this study, we demonstrated that *AaeIAP1* gene expression was induced to significantly higher levels in response to both cold and heat shock, suggesting that *AaeIAP1* might play an important role in stress-induced apoptosis.

Several IAP genes have been demonstrated to be capable of preventing programmed cell death induced by UV irradiation. For example, when the IAP gene from *Orgyia pseudotsugata* nuclear polyhedrosis virus was overexpressed in insect cells, the UV radiation-induced apoptosis was blocked (Manji et al. 1997). However, information on IAP gene regulation in organisms after UV irradiation is unknown. Our study, for the first time, demonstrated that UV irradiation significantly increased the expression of *AaeIAP1* in *Ae. aegypti.* This result is consistent with previous human cancer cell studies on expression level of survivin (an IAP homolog in human), which demonstrated that survivin expression level is significantly higher in UV-induced melanoma cells than in normal cells (Raj et al. 2008). The up-regulation of *AaeIAP1* followed by UV radiation strongly suggests that *AaeIAP1* play a functional role in UV radiation-induced apoptosis.

The primary approach used for mosquito control has mainly relied on pesticides. Because of its low mammalian toxicity, pyrethroid insecticides are widely used to impregnate bed nets and indoor residual spray programs in an effort to control mosquitoes. However, frequent use of pyrethroids has resulted in development of resistance in field populations (Jinfu 1999, Wang 1999). Gene regulation has been extensively studied in pesticide resistant mosquitoes. For example, in an insecticide resistant strain of *Anopheles stephensi* Liston glutathione *S*-transferase transcripts and cytochrome P450 gene expression are reported to be up-regulated (Vontas et al. 2007). However, IAP gene regulation upon pesticide treatment has never been studied. Our study, for the first time, demonstrated that *AaeIAP1* gene expression was significantly increased in response to permethrin treatment, suggesting that *AaeIAP1* might play a pivotal role in stress-induced apoptosis.

In conclusion, the expression of *AaeIAP1* transcript was tightly regulated during the physiological development in *Ae. aegypti*, with significantly higher levels in the pupal and adult stages compared with the larval stage. Furthermore, *AaeIAP1* expression levels were increased significantly in response to different environmental stresses (e.g., low and high temperatures, UV irradiation, and permethrin treatment), suggesting that *AaeIAP1* not only plays an important role in the physiological process of the development of *Ae. aegypti* but also plays a pivotal role in stress-induced apoptosis. This study may provide information useful for designing novel control strategies for mosquitoes.

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