HEAT-SHOCK MORTALITY AND INDUCED THERMOTOLERANCE IN LARVAE OF THE MOSQUITO *ANOPHELES ALBIMANUS*

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ABSTRACT. Temperature effects on *Anopheles albimanus* larval survival were investigated. Larvae were exposed to 30 min heat shocks at various temperatures. Almost no mortality was observed at 40°C, but was complete at 43°C. Increased larval thermotolerance could be induced by higher rearing temperature or by a 30 min exposure to 37°C.

INTRODUCTION

Heat-induced thermotolerance has been observed in numerous Diptera, e.g., *Drosophila melanogaster* Meigen (Alahiotis and Stephanou 1982, Berger and Woodward 1983, Singh and Lakhotia 1988), a tropical species of *Chironomus* (Nath and Lakhotia 1989), and the medfly, *Ceratitis capitata* Wiedemann (Stephanou et al. 1983a). Generally this is demonstrated by exposing insects to a relatively mild heat shock before exposure to temperatures in the lethal range. Alternatively, insects are reared at various temperatures before the lethal exposure. The results of both types of experiments are consistent with increased survival as a consequence of previous exposure to elevated temperatures.

In this study, similar experiments were conducted for the tropical mosquito *Anopheles albimanus* Wiedemann. We are interested in inducible genes which allow it to increase its survival during heat stress, and such studies are facilitated by the extensive genetic research on this particular species. Toward that end, we asked at what temperature does heat-induced mortality occur, how broad is the range, whether it is affected by the rearing temperature or prior exposure to sublethal heat shock, and whether more extreme sublethal heat shocks produce greater thermotolerance.

MATERIALS AND METHODS

Heat-shock mortality in relation to rearing temperature: Anopheles albimanus larvae from the USDA Medical and Veterinary Entomology Research Laboratory main colony were reared at 25 or 30°C (± 0.5°C) from egg hatch to the fourth instar on a diet of 2 parts of TetraMin Baby-E Fish Food® to 1 part brewer's yeast (Benedict et al. 1979). One hundred mid- to late fourth stage larvae were counted into each of 6 treatment containers consisting of 100 ml plastic beakers, the bottom of which had been cut off and replaced with fine plastic screen. These were transferred to identical foam ice chests containing approximately 5 liters of municipal supply water adjusted to 37, 38.5, 40, 41.5 and 43°C for the 25°C rearing tests, or 38.5, 40, 41.5 43 and 44.5°C for the 30°C rearing test. Controls for the heat-lethality tests were larvae counted and handled the same as the heat-treated larvae, but transferred to identical chests filled with 25 or 30°C water, depending on the original rearing temperature. The temperature in these chests was maintained within 0.5°C by stirring the water, and adding warm water every 5 to 10 minutes. Larvae were held at the different temperatures for 30 min and then transferred back to water at 25 or 30°C for 30 min, at the end of which time dead larvae were counted. These experiments were repeated 3 times.

Heat-induced heat tolerance experiments: All larvae for these tests were handled the same as those above and were reared at 25°C. Sublethal heat shocks were administered at 28, 33, or 37°C for 30 minutes. The control was similar but transferred to 25°C. Each of these temperature groups contained 5 groups of 100 larvae. At the end of the heat-treatment period, the larvae were transferred back to 25°C for 30 minutes. One beaker from each of the groups was transferred to 25, 40, 41.5, 43, or 44.5°C. They were held there for 30 min and then once again transferred back to 25°C for 30 min, at the end of which time dead larvae were counted. These experiments were repeated 3 times.

Data analysis: Mortality data were transformed by an angular transformation, the arcsin of the square root of the percent mortality. Analysis of variance (ANOVA) and Duncan's multiple range test were used to compare the transformed mortalities using the SAS procedure ANOVA. The main effects of the rearing temperature experiments were replicate, rearing and lethal-range temperature. Only the temperatures that were used to treat both the 25 and 30°C larvae (38.5, 40, 41.5 and 43°C) were used

Table 1. Average numbers of larvae killed in rearing-temperature experiments.

Rearing temp.	Treatment temperature (°C ± 0.5°C)							
	25.0	30.0	37.0	38.5*	40.0	41.5	43.0	44.5
25.0	ND	0**	0	0.3	8	70.7	100	ND
30.0	ND	0	0	0	0	8.7	98.7	100

* Only data below the underlined temperatures were used for ANOVA.
** Mean no. larvae killed from 3 replicates.

for statistical comparisons. For the second set of experiments, the main effects considered were replicate, preshock and lethal-range temperature. Transformed mortalities of larvae reared at 25°C in the first set of experiments and controls in the second set that were mock preshocked at 25°C were compared using the SAS procedure TTEST. The significance level for all statistics was 0.05.

RESULTS AND DISCUSSION

Heat-shock mortality in relation to rearing temperature: A set of experiments was designed to determine larval mortality at various temperatures, and whether tolerance to these temperatures could be increased by rearing larvae at a higher temperature. Two rearing temperatures, 25 and 30°C, were chosen. These temperatures promote high survival and reasonable development times. Table 1 shows the raw mortality data and a graphical representation is shown in Fig. 1. Duncan groupings are shown in Table 2.

Larvae reared at 25°C were killed in significantly higher numbers than those reared at 30°C

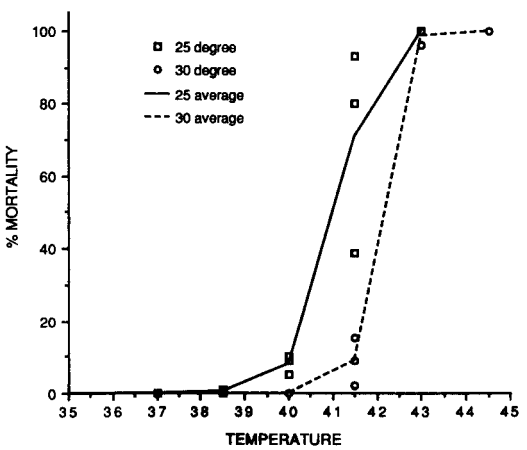

Fig. 1. Rearing-temperature experiments. The numbers of larvae killed when reared at 2 temperatures are plotted against lethal-range temperature.

Table 2. Duncan's multiple range test grouping for rearing temperature (°C) experiments.

Effect						
Lethal temperature	38.5	40.0	41.5	43.0		
Grouping[a]	⊢——⊣	⊢——⊣	⊢—⊣	⊢—⊣		
Replicate	1	3	2	5	6	4
Rearing temperature	25	25	25	30	30	30
Grouping	⊢——⊣		⊢——⊣			
		⊢——⊣		⊢——⊣		
Rearing temperature	25.0	30.0				
Grouping	⊢——⊣	⊢——⊣				

[a] Continuous bars join variables with the same Duncan grouping.

($P > F = 0.0001$). This demonstrates that raising the rearing temperature can decrease sensitivity to heat, and that all mortality occurs in a very narrow temperature range from 40 to 43°C.

Lethal temperature in general was a significant variable ($P > F = 0.0001$) although mortality at 38.5°C was not significantly different from that at 40°C (Table 2). Replicate variation was also significant ($P > F = 0.0007$) due to uncontrolled variables affecting the mortality in the steep slope range of the response curve. Significant differences occurred between the first and second replicates among 25°C-reared larvae (replicates 1–3). Replicate tests of larvae reared at 30°C (replicates 4–6) were not significantly different. No control mortality occurred in these or the following experiments.

Preshock-induced heat-tolerance experiments: The second set of experiments determined the effect of a brief sublethal heat shock on heat sensitivity. The sublethal shock temperatures chosen were based on the results of the first sets of experiments above. The highest temperature at which no mortality had been observed was 37°C, so extreme shocks were administered at this temperature. Moderate shocks were administered at 33°C, and mild shocks at 28°C. The raw mortality data are graphed in Fig. 2. Table 3 shows the raw data and Table 4 the Duncan groupings.

Larvae preshocked at 37°C were significantly more resistant to heat-killing than those shocked at 28°C or controls ($P > F = 0.0053$). The 28 and 33°C groups show a trend toward

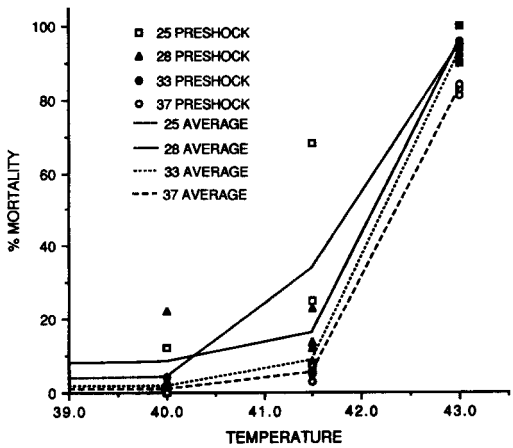

Fig. 2. Preshock experiments. The numbers of larvae killed when preshocked at 3 temperatures and the control values are plotted against lethal-range temperature.

Table 3. Average numbers of larvae killed in preshock-induced heat-tolerance experiments.

Preshock temp.	Treatment temp. (°C ± 0.5°C)			
	25.0	40.0	41.5	43.0
25.0	0*	4	33.7	95
28.0	0	8.3	16.3	95.7
33.0	0	1.7	8.7	92.7
37.0	0	0.7	5.3	82.7

* Average of 3 replicates.

Table 4. Duncan's multiple range test grouping for preshock-induced heat-tolerance experiments (temperature in °C).

Effect				
Lethal temperature	40.0	41.5	43.0	
Grouping[a]	⊢⊣	⊢⊣	⊢⊣	
Replicate	1	2	3	
Grouping	⊢⊣			
		⊢⊣		
Preshock temperature	25.0	28.0	33.0	37.0
Grouping	⊢			
			⊢	⊣

[a] Continuous bars join groups with the same Duncan grouping.

decreased heat sensitivity which might have been statistically significant, if the variability had been lower. However, once again in the second set of experiments, statistically significant differences were observed between the first and third replicates ($P > F = 0.0145$), probably for the same reasons suggested above.

Larvae were reared at 25°C and heat treated similarly in the rearing temperature and preshock experiments, allowing comparison of the mortality. This comparison would allow detection of significant temporal changes in the heat sensitivity of the larvae. This would also reveal effects of handling differences between the first and second experiments. Those possibilities were not realized because the amount of heat-caused mortality in the 25.0°C group of the first experiments and the 25°C controls of the second set were not significantly different (t-test, $P > F' = 0.95$).

These experiments show that not only does rearing larvae at higher temperatures increase their resistance to heat-killing, but a relatively brief 30 min exposure to 37°C also increases their heat resistance. Some thermotolerance can probably be induced by the slightest of temperature elevations. However, in these experiments, the sensitivity is limited by replicate variation due to uncontrolled variables.

These observations of inducible thermotolerance after a brief shock, or as a result of different rearing temperatures, are similar to those made in *D. melanogaster* (Alahiotis and Stephanou 1982, Berger and Woodward 1983, Singh and Lakhotia 1988), a tropical species of *Chironomus* (Nath and Lakhotia 1989), and the medfly *Ceratitis capitata* (Stephanou et al. 1983a). The lethal temperature range that we have observed is also similar to that seen in the above references, although direct comparisons are difficult to make due to differences in the treatment methods and periods after treatment at which lethality was determined. In the experiments reported here, additional delayed mortality might have occurred among larvae that were counted as survivors.

What is the physiological basis for thermotolerance? Although thermotolerance is undoubtedly complex, increased thermotolerance is positively correlated with increased synthesis of heat-shock proteins (reviewed by Craig 1985). For example, *D. melanogaster* genetic strains have been selected for cold or warm rearing conditions (Stephanou et al. 1983b, Alahiotis and Stephanou 1982). The cold-selected strain is more sensitive than the warm-selected to heat-killing when reared similarly. The sensitive strain produces lower levels of heat-shock proteins than does the tolerant. Other experiments have shown that this genetic effect can be mimicked merely by rearing the same strain at 2 temperatures (Singh and Lakhotia 1988). Cold-reared *D. melanogaster* are more sensitive to heat killing than those reared warmer. Similarly, medflies that have been preshocked have higher levels of heat-shock proteins and thermotolerance than larvae not shocked (Stephanou et al. 1983a). The onset of thermotolerance in *D. mel-*

anogaster embryos occurs at gastrulation, the same stage at which they are able to synthesize heat-shock proteins (Bergh and Arking 1984). Finally, when *D. melanogaster* cells are treated with ecdysone, which is known to induce synthesis of the small heat-shock proteins, thermotolerance increases (Berger and Woodward 1983, Berger 1984). Likewise, immature stages of whole animals have greater thermotolerance during periods of higher ecdysone titer.

These types of experiments, though suggestive, are not as conclusive as data from bacteria and yeast showing that deletion mutants for heat-shock protein genes are unable to grow at elevated, or sometimes even normal temperatures, but can grow at reduced temperatures (Craig 1985).

Seasonal variation in the levels of heat-shock protein synthesis probably occurs in mosquitoes. This has been observed in natural populations of *Chironomus* (Nath and Lakhotia 1989). These authors observed seasonal and temperature-related variation in chromosome puffing and in heat-shock protein inducibility. Heat-shock induction of puffs and proteins was greater in larvae that were laboratory reared at a constant temperature than in those that had been exposed to warmer natural conditions and were already synthesizing heat-shock proteins. The field-collected larvae were also less sensitive to heat killing than the constant-temperature laboratory-reared larvae.

Thermoprotection is a common necessity for mosquitoes, particularly where tropical larval habitats are in exposed sites where daily and seasonal temperature fluctuations occur. These experiments demonstrate that mechanisms exist in *An. albimanus* for increasing its survival under variable temperature conditions.

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