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THE EFFECTS OF LOW TEMPERATURES ON EGGS OF *AEDES AEGYPTI* (L.) [1]

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INTRODUCTION

Low winter temperatures are a limiting factor in the survival of many species of insects and, in many instances, restrict their geographical range. An excellent example of such a restricted range is that of *Aedes aegypti* (L.). Christophers (1960) states that the species is probably the only mosquito that, with human assistance, is spread around the globe. He points out that in spite of this wide distribution, it is limited by latitude (45° N and 35° S); distance from the sea; desert conditions; and isolation from human intercourse. Primarily, the northern and southern distributions appear to be related to temperature. There appears to be, with a few exceptions, a striking correlation between the mean isotherm of approximately 50° F for January in the northern hemisphere and July in the southern hemisphere. One of the more notable exceptions is in the eastern portion of the United States where it has been found as far north as Boston, Massachusetts (Christophers, 1960).

Any insect in a cold climate requires some form of protection against low winter temperatures. This may be the insulating protection of its environment or the ability of the insect to undercool (Salt, 1950). The ability to undercool confers cold-hardiness to an insect species (Salt, 1953, 1961), and many species possess an inherent cold-resistance which enables them to survive extremely low temperatures (Salt, 1956).

This paper reports a portion of the studies conducted by the Technical Development Laboratories (TDL) on the possible inherent cold-resistance in *Ae. aegypti*. Specifically, these experiments were undertaken to determine: (1) if *Ae. aegypti* from different localities (potentially different gene pools) are equally tolerant to low temperatures during the egg stage; (2) if *Ae. aegypti* eggs are capable of surviving temperatures at or near freezing and, if so, for what period of time; and (3) what effect sublethal low temperature exposure has on subsequent larval survival and adult emergence.

MATERIALS AND METHODS

I. SOURCE OF TEST SPECIMENS. Eggs of strains of *Ae. aegypti* from Puerto Rico; Cucuta, Colombia; Trinidad, B.W.I.; Camp Detrick, Maryland; St. Thomas, Virgin Isles; Pensacola, Florida; Lagos, Nigeria; Sudan; Galveston, Texas; Bangkok, Thailand; Queens, Ontario; and

[1] From the Technical Development Laboratories, Malaria Program, Center for Disease Control, Public Health Service, Health Services and Mental Health Administration, U. S. Department of Health, Education, and Welfare, Savannah, Georgia 31402.

Guelph, Ontario, were obtained from various investigators. The majority of these eggs came from already established colonies, but represented different gene pools. Colonies of each strain were established at TDL to provide test material. At the conclusion of preliminary experiments, only the first six strains listed were retained for further study.

The colonies were maintained in screened cages 18" x 18" x 23"; fed 10 percent sucrose daily; held at 80° F and 75 to 80 percent R.H.; and offered a rabbit as a blood source daily. The females oviposited on strips of wet paper toweling which were placed in the colony cages overnight, removed and placed, still wet, in a sealed storage container for 48 additional hours. They were then air dried at 80° F and 75 to 80 percent R.H. and stored at 80° F and saturated humidity until needed for routine colony maintenance.

II. PRE-EXPOSURE HANDLING. In those tests with eggs less than 24 hours old, fresh oviposition strips were placed in the colony cages at approximately 4:30 p.m. and removed at approximately 8:00 a.m. the next morning. Excess water was removed and the oviposition strips were then cut into small pieces, each containing about 100 eggs and placed, eggs up, upon a damp cellulose sponge and sealed in a plastic container which was placed in the low temperature chambers. These eggs had a possible age range, at the time the chilling started, of from 1 to 17 hours.

In those tests with 3-day-old eggs, the wet strips were removed from the sealed storage container as described under I, the excess water removed by blotting, and then exposed to low temperatures as described under II. In all tests with egg strips older than 3 days, the strips were cut into pieces of about 100 eggs while dry, placed dry upon the damp cellulose sponge, and sealed in the plastic container for exposure.

In early tests, the egg strips were merely placed in sealed plastic containers while working in an environment of 80° F and 80 percent R.H. The atmosphere within the closed container became saturated at the ambient temperature. When these containers were then placed in the cold chambers, the relative humidity within the container became supersaturated and the water condensed on the walls of the container and on the eggs themselves. Technically, these eggs were at that point "immersed," and many began to hatch. Those that obviously hatched did not pose too great a problem, for when examined microscopically they were quite obvious and could be discounted in the evaluation. However, microscopic examination revealed that many eggs only partially hatched during the exposure period, and a very minute rupture of the operculum occurred at the line of dehiscence. In many cases the line of fracture was so minute that it was overlooked; in some cases the eggs with a slightly cracked operculum seemed to hatch and produce living larvae and at other times did not. Little consistency in test data among replicates could be obtained. These problems were eliminated by placing the egg strips on a moist cellulose sponge within the exposure container. As the water condensed on the oviposition strips, it was drawn off by the sponge. Microscopic examination revealed that the eggs did not hatch nor did the opercula crack during the exposure. Thereafter consistent test data and reproducible results were obtained among replicates.

III. LOW TEMPERATURE EXPOSURE. In preliminary tests the eggs were refrigerated for 1, 2, 3, 4 and 7 days at temperatures of 5, 24, 27, 31, 34, 37, 40, 43, 46, 49, 52, 55, 58 and 61° F. The data from these tests indicated that the temperatures between 31° and 40° F would be most suitable for our studies since temperatures below 21° F caused almost total mortalities and temperatures above 40° F caused essentially no mortality. Most of the tests were conducted at 37° F and for exposure periods of 1, 2, 3, and 4 days and 1, 2, 3, 4, 6 and 8 weeks.

The eggs were exposed in commercial

freezers that had had their thermostats modified so that the desired temperature ranges could be obtained with the "on-off" deviations limited to less than 1° F. The freezers had a fan for continuous mechanical movement of the air within the chamber. Even with these mechanical aids, thermistors at various points within the chamber showed that the temperatures at various positions within the chamber varied as much as 1° F. To nullify the possible difference in effect of such temperature variation, the plastic containers were arranged systematically so that one container of each age group occupied each position within the chamber. Chamber temperatures were continuously monitored with thermistors (Y.S.I. probe 4010[2] which were connected via a multiple switch box to remote indicating thermometers (Y.S.I. Telethermometer, model 42–SF) which in turn were connected to a dual channel recorder (Y.S.I. model 81). This equipment permitted fluctuations as small as 0.1° F to be accurately recorded at 1-second intervals.

IV. POST EXPOSURE HANDLING. In one series of tests in which the eggs were exposed to 5, 31, 34 and 37° F, the eggs were removed at intervals of 1, 2, 3 and 4 days, and 1, 2, 3, 4, 6 and 8 weeks and microscopically examined under ambient temperature for evidence of hatching during exposure. They were then immersed for 24 hours in hatching medium prepared the day before by adding 100 mg of laboratory chow and 50 mg of powdered brewer's yeast to 1 liter of water. All larvae produced were counted and reared in enamel trays providing at least 1 sq cm feeding surface and at least 1 ml of water per larva. They were reared in a controlled environment of 80° F and 80 percent R.H., and fed ground Purina laboratory chow that had been passed through a 40-mesh sieve. They were fed 0.15, 0.3 and 0.4 mg of food/larva on days 0, 1 and 2, respectively, and 0.6 mg/larva daily thereafter until the first day of pupation.

In another series of tests in which the eggs were exposed to 5, 31, 34 and 37° F for only 1, 2, 3, 4 and 7 days, the eggs were removed and examined, but were placed in the hatching medium for 7 days. On days 1, 2, 3, 4 and 7, those larvae that had hatched during the previous time interval were removed, counted and reared as previously described.

Each test consisted of three replicates for each egg age; each temperature; and each exposure period. All tests were repeated no less than three times.

RESULTS

I. EGGS LESS THAN 24 HOURS OLD. None of the eggs of any strain that were less than 24 hours old when placed in temperatures below 37° F hatched when subsequently immersed for 24 hours in hatching media, regardless of the length of the exposure period.

After 1 day of exposure to 37° F less than 1 percent of the Camp Detrick (CD) strain and none of the four remaining strains hatched. None of the CD larvae lived. After 2, 3 and 4 days of exposure, no hatch occurred in any of the strains but after 1 week of exposure, one egg of the Pensacola #1 (P#1) strain hatched, but did not complete larval development. None of the eggs of the remaining strains hatched. After 2, 3, 4, 6 and 8 weeks of exposure, one egg hatched of the Pensacola #2 (P#2) strain from the group chilled for 6 weeks.

Because there was a possibility that embryonic development had merely been retarded by the temperatures of 31, 34 and 37° F, and not killed, the exposures through 7 days were repeated, but the eggs were kept in hatching media for 7 days instead of 24 hours. Daily observations revealed that none of the eggs hatched.

II. EGGS 3 DAYS OLD. With eggs of all

[2] Use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service or the U. S. Department of Health, Education, and Welfare.

TABLE 1.—Percentage of 3-day-old *Aedes aegypti* eggs hatching after exposure to 37° F for time intervals of:

Strain	1 day	2 days	3 days	4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	8 weeks	Mean
Puerto Rico	81	66	68	45	19	9	1	0	0	0	29
St. Thomas	77	67	57	44	26	7	6	0	0	0	28
Pensacola #1	65	64	60	38	17	8	6	1	0	0	26
Pensacola #2	72	63	51	34	19	6	2	0	0	0	25
Camp Detrick	59	63	51	32	19	4	3	2	0	0	23
Trinidad	63	54	61	50	20	7	0	0	1	0	26
Cucuta	68	60	62	45	21	6	3	0	0	0	26
Mean	69	62	59	41	20	7	3	<1	<1	0	26

Each percentage represents a minimum of three separate tests, each consisting of three replicates of approximately 100 eggs each.

strains that had been kept moist for approximately 72 hours and maintained at approximately 80° F (the normal period required for completing embryonic development) and exposed to 31, 34 and 37° F for the standard exposure periods, the lower temperatures (31, 34° F) resulted in less hatch at all exposure periods. Since the data for all three temperatures were similar, only those from the 37° F exposure are reported.

The data (Table 1) showed that the eggs exposed for 3 days or less had 50 percent or greater hatch. Exposures of 1 week generally resulted in about 20 percent hatch and exposures of 2 weeks or more resulted in less than 10 percent hatch.

At the bottom of Table 1 is shown the mean hatch of all strains for each exposure period so that the individual response of any given strain may be related to it. Comparison of these data reveals that the greatest deviation from the mean percent hatch occurred among those eggs exposed for 4 days or less.

In the extreme right column of Table 1 is the mean hatch for each strain for the entire exposure of 8 weeks. Although the PR and StT strains have the most hatch (29 and 28 percent, respectively), and the CD strain the least (23 percent), the difference is too small to be meaningful.

Because there was a possibility that embryonic development or that the time required for hatching had merely been altered, the low temperature exposures up to 7 days were repeated, and these eggs were kept in the hatching media for 7 days instead of 24 hours. Daily observations revealed (Table 2) that some eggs did have a delayed hatch, but that the percent hatching after the first 24 hours was small. The data for the individual strains are not presented since they revealed no marked difference among the various strains.

In those tests in which the eggs were exposed, hatched and then reared to adults, the data (Table 3) revealed that the P#1 and CD strains produced the least number of adults. The StT, PR and Cucuta (Cu)

TABLE 2.—Mean percent of daily hatch of all strains of 3-day-old eggs of *Aedes aegypti* exposed to 37° F for periods up to 1 week and kept in hatching media for 1 week.

Days in hatching media	Days of exposure				
	1	2	3	4	7
1	64	58	58	37	18
2	2	4	2	9	2
3	<1	3	2	2	2
4	0	1	<1	2	2
7	0	0	<1	<1	2
Total	66	66	63	50	26

Each percentage represents a minimum of three separate tests, each consisting of three replicates of approximately 100 eggs each.

strains produced the most. When this information is compared with the hatch data from Table 1, the extremes are apparent in three strains: the PR and StT strains, with their larger hatch and subsequent adult production; and the CD strain with its smaller hatch and corresponding smaller adult production. These data would lead one to assume that the larger hatch of the PR and StT strains was obviously the reason for the larger percentage of adults produced from such egg exposures. The data in Table 4, however, do not fully bear out such an assumption, since they show that of the larvae that hatched from 3-day-old eggs exposed to 37° F for all periods up to 8 weeks, the mean percentage of the PR strain that completed development to the adult stage was 71, whereas that of the CD strain was 61. The CD strain also was not the strain having the lowest mean adult production from hatched larvae, which suggests that although the eggs of the CD strain are not as tolerant to low temperature exposure as are those of some other strains, those larvae of the CD strain that hatch are equally as capable of surviving.

The adults from the above tests were killed, sexed and counted. Females outnumbered males, with a mean percentage for all strains and all exposures of approximately 40 percent male. The CD strain produced the fewest males (37 percent) and the StT and PR strains pro-

TABLE 3.—Percentage of all 3-day-old *Aedes aegypti* eggs exposed to 37° F for various time intervals that subsequently completed development to the adult stage.

Strain	1 day	2 days	3 days	4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	8 weeks	Mean
Puerto Rico	49	61	41	47	15	5	1	0	0	0	22
St. Thomas	64	53	42	56	15	6	1	0	0	0	24
Pensacola #1	39	40	46	31	12	2	4	1	0	0	17
Pensacola #2	57	47	41	40	7	3	0	0	0	0	19
Camp Detrick	29	30	13	32	11	2	<1	2	0	0	12
Trinidad	49	30	50	62	10	2	0	0	0	0	20
Cucuta	63	45	50	42	15	1	1	0	0	0	22
Mean	50	44	40	44	12	3	1	<1	0	0	19

Each percentage represents a minimum of three separate tests, each consisting of three replicates of approximately 100 eggs each.

TABLE 4.—Percentage of hatched larvae (from 3-day-old eggs of *Aedes aegypti* exposed to 37° F for various time intervals) that completed development to the adult stage.

Strain	1 day	2 days	3 days	4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	8 weeks	Mean
Puerto Rico	52	78	65	85	60	56	100 *	..	..	..	71
St. Thomas	72	73	67	93	41	86	17	..	..	..	64
Pensacola #1	55	54	69	77	66	25	67	100 *	..	..	63
Pensacola #2	63	59	82	89	32	50	0	..	..	..	53
Camp Detrick	62	64	36	94	69	50	10 *	100 *	..	..	61
Trinidad	73	58	72	92	42	29	..	..	0	..	52
Cucuta	82	56	77	70	56	17	50	..	..	..	58
Mean	66	63	67	86	51	45	49	100 *	0	..	58

Each percentage represents a minimum of three separate tests, each consisting of three replicates of approximately 100 eggs each.
* These percentages represent larvae from tests in which egg hatch was 3 percent or less.

duced the most (46 and 44 percent males, respectively).

III. EGGS 14 DAYS OLD. In the tests using 14-day-old eggs that had been stored at 80° F and saturated humidity, the results were evaluated in the same manner as those of the 3-day-old eggs. Tabular data are not presented for each of the procedures except when there were major variations from the data obtained with 3-day-old eggs.

In those tests in which 14-day-old eggs were exposed to 37° F for periods of 1, 2, 3 and 4 days and 1, 2, 3, 4, 6 and 8 weeks,

TABLE 5.—Mean percentages of 3- and 14-day-old *Aedes aegypti* eggs * hatching after exposure to 37° F for all exposure periods through 8 weeks.

Strain	3 days old	14 days old
Puerto Rico	29	38
St. Thomas	28	37
Pensacola #1	26	37
Pensacola #2	25	34
Camp Detrick	23	34
Trinidad	26	24
Cucuta	26	37
Mean	26	34

* Approximately 63,000 eggs for each age group.

the Trinidad (T) strain was the only one demonstrating any obvious difference in response, since it consistently had about a 10 to 15 percent smaller hatch at most exposure periods. When comparing the mean percent hatch for all exposure periods with similar data for the 3-day-old eggs (Table 5), it was evident that the 14-day-old eggs had a proportionate increase of approximately 31 percent.

When the mean percent hatch for all strains of 3- and 14-day-old eggs was compared (Table 6) for each exposure period at 37° F, the most apparent difference was the greater hatch of the 14-day-old eggs following exposure periods of more than 1 week. The data in Table 6 also show that the 14-day-old eggs had a hatch of 10 percent or more following exposure periods for as long as 4 weeks, whereas the 3-day-old eggs had no hatch of 10 percent or more following exposure periods of more than 1 week. Six weeks

TABLE 6.—Mean percent hatch (all strains) of 3- and 14-day-old eggs after exposure to 37° F.

Exposure	3 days old	14 days old
1 day	69	73
2 days	62	61
3 days	59	61
4 days	41	61
1 week	20	34
2 weeks	7	32
3 weeks	3	12
4 weeks	<1	10
6 weeks	<1	0
8 weeks	0	0

of exposure to 37° F were required to reduce the hatch of 14-day-old eggs to zero, but the hatch of 3-day-old eggs was reduced to essentially zero after 4 weeks of exposure.

In those tests in which 14-day-old eggs were exposed, hatched and reared to adults, the mean percent (Table 7) of all strains and all exposure periods that emerged as adults was 26, whereas similar data for the 3-day-old eggs revealed a mean production of 19 percent. The PR and P #2 strains produced the larger percentage (29 percent) and the T strain the smallest (18 percent). Except for the smaller production by the T strain, no marked variations among the strains were noted. Seventy-six percent of the larvae that hatched from all exposures and all strains completed their development and became adults. This figure approaches the control production figure of 80 percent, indicating that most larvae that do hatch following low temperature exposure as 14-day-old eggs are essentially capable of normal development. This fact is in marked contrast to the data from exposure of 3-day-old eggs (Table 4) in which the percent of larvae hatched completing development was 58.

TABLE 7.—Mean percent of 3- and 14-day-old eggs of *Aedes aegypti* exposed to 37° F that completed development to the adult stage.

Exposure	3 days old	14 days old
1 day	50	53
2 days	44	51
3 days	40	43
4 days	44	44
1 week	12	30
2 weeks	3	18
3 weeks	1	10
4 weeks	<1	8
6 weeks	0	0
8 weeks	0	0
Mean	19	26

The adults produced from the 14-day-old eggs exposed to 37° F were killed, sexed and counted. The data from all exposure periods and strains (Table 8) show that 39 percent of all adults were males which is a ratio similar to that for the 3-day-old eggs. Examination of the data for individual strains revealed no marked variation from the mean production among the strains.

Examination of the data for each exposure period does reveal a reduction in the mean percent of adult males produced from 14-day-old eggs exposed for 2 weeks. Examination of other data for the 2-week exposures indicated a normal hatch (32 percent) for that temperature and exposure period, but only 56 percent of the larvae that hatched survived to become adults. This rate was the lowest for all exposure periods and was in marked contrast to the maximum mean survival rate of 88 percent (1-week exposures) and to the mean survival of all strains and all exposure periods of 76 percent.

In all of the tests, control eggs held for periods as long as 12 weeks showed a hatch of approximately 90 percent with approximately 80 percent of the resultant larvae completing their development.

DISCUSSION

In these studies, the strains used had been separated and isolated, either physically or geographically, for a period of time long enough to have developed unique inheritable characteristics if such definite factors existed. The T strain, originally a DDT-resistant strain from Trinidad, B.W.I., had been under heavy DDT selection in the laboratory for many years and was highly resistant to that

TABLE 8.—Percent of male and female adult *Aedes aegypti* produced from 14-day-old eggs * exposed to 37° F for each time interval.

Strain	1 day	2 days	3 days	4 days	1 week	2 weeks	3 weeks	4 weeks	6 weeks	8 weeks	Mean
Puerto Rico ♂	37	31	29	37	33	26	37	33	..	..	33
St. Thomas ♂	43	36	45	58	39	27	47	59	..	..	44
Pensacola #1 ♂	36	41	43	48	42	28	33	40	..	..	39
Pensacola #2 ♂	34	29	53	50	53	23	37	40	..	..	40
Camp Detrick ♂	25	42	54	50	34	36	45	36	..	..	40
Trinidad ♂	45	35	35	41	40	33	25	..	..	..	36
Cucuta ♂	46	50	45	44	23	25	33	40	..	..	38
Mean ♂	38	38	43	47	38	28	37	41	..	..	39
Puerto Rico ♀	63	69	71	63	67	74	63	67	..	..	67
St. Thomas ♀	57	64	55	42	61	73	53	41	..	..	56
Pensacola #1 ♀	64	59	57	52	58	72	67	60	..	..	61
Pensacola #2 ♀	66	71	47	50	47	77	63	60	..	..	60
Camp Detrick ♀	75	58	46	50	66	64	55	64	..	..	60
Trinidad ♀	55	65	65	59	60	67	75	..	..	..	64
Cucuta ♀	54	50	55	56	77	75	67	60	..	..	62
Mean ♀	62	62	57	53	62	72	63	59	..	..	61

* Approximately 63,000 eggs.

chemical. The PR strain was a dieldrin-resistant strain which had not been under dieldrin selection. The C strain and the StT strain were moderately resistant to DDT and dieldrin. The CD strain was a susceptible laboratory reference strain that had been in colony production for many years. The two Pensacola strains had just been colonized from field-collected material in Pensacola, Florida, and were moderately resistant to DDT.

The data show that: (1) eggs subjected to temperatures below 37° F before embryonic development had been completed were incapable of hatching, even when kept in hatching media for periods as long as 1 week after exposure; (2) eggs in which the embryo had completed its development were capable of surviving, though not in normal numbers; (3) eggs which had entered the dormant phase (14-day-old eggs) were more capable of surviving than were those that had just completed their embryonic development; and (4) eggs destined to develop into females were more capable of surviving than those for males. As one would expect, the longer the duration of low temperature exposure, the fewer the eggs that survived. The studies indicated that even at 37° F, a temperature well above freezing, fully embryonated eggs failed to survive 6 weeks exposure. These observations suggest that the eggs do not have an efficient mechanism for supercooling.

The observations from the studies with fully embryonated eggs revealed that larvae hatching from those eggs which had completed embryonic development just prior to cold exposure (3 days old) had been affected by the low temperature and only 58 percent of them were capable of completing larval development. However, the larvae hatching from those eggs which were fully embryonated and dormant (14 days old) when exposed to cold were apparently little affected by the exposure, for the percent completing their development was similar to the normal production figures.

The overall conclusions indicated that of the seven populations tested no one population differed greatly from any other in its ability to survive low temperatures during the egg stage. Whether one or all of the populations could change if subjected to prolonged low temperature selection is yet to be determined. The data would suggest that there were some individuals among all of the strains more capable of surviving low temperatures than others. Based on an analogy to individuals in a population being less susceptible to an insecticide and by selection pressure thereby developing an insecticide resistant strain, it appears conceivable that a strain resistant to low temperatures could be developed.

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