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THE ABILITY OF AEDES (STEGOMYIA) AEGYPTI TO ADAPT TO LOW TEMPERATURES

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THE ABILITY OF AEDES (STEGOMYIA) AEGYPTI

TO ADAPT TO LOW TEMPERATURES

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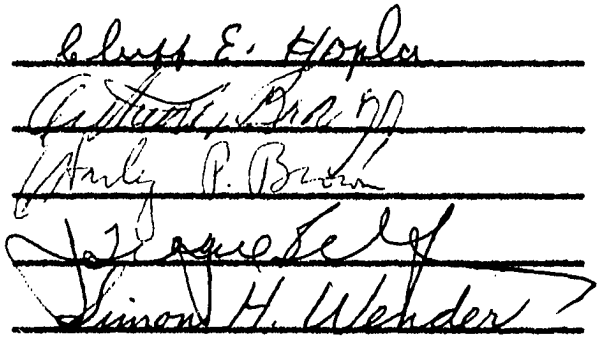

DISSERTATION COMMITTEE

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THE ABILITY OF AEDES (STEGOMYIA) AEGYPTI TO ADAPT TO LOW TEMPERATURES

CHAPTER I.

INTRODUCTION

The discovery that mosquitoes are vectors of disease is beyond doubt responsible for the tremendous amount of research on them. Important in this regard is the tribe Anophelini, due to their role as vectors of malaria, and the tribe Culicini, especially the genera Culex and Aedes. This applies especially to the single species, Aedes aegypti, because its distribution is almost world-wide within the tropical and subtropical zones and it serves as a vector not only for viruses of Yellow Fever and/or Dengue throughout much of its distribution but also Wuchereria bancrofti.

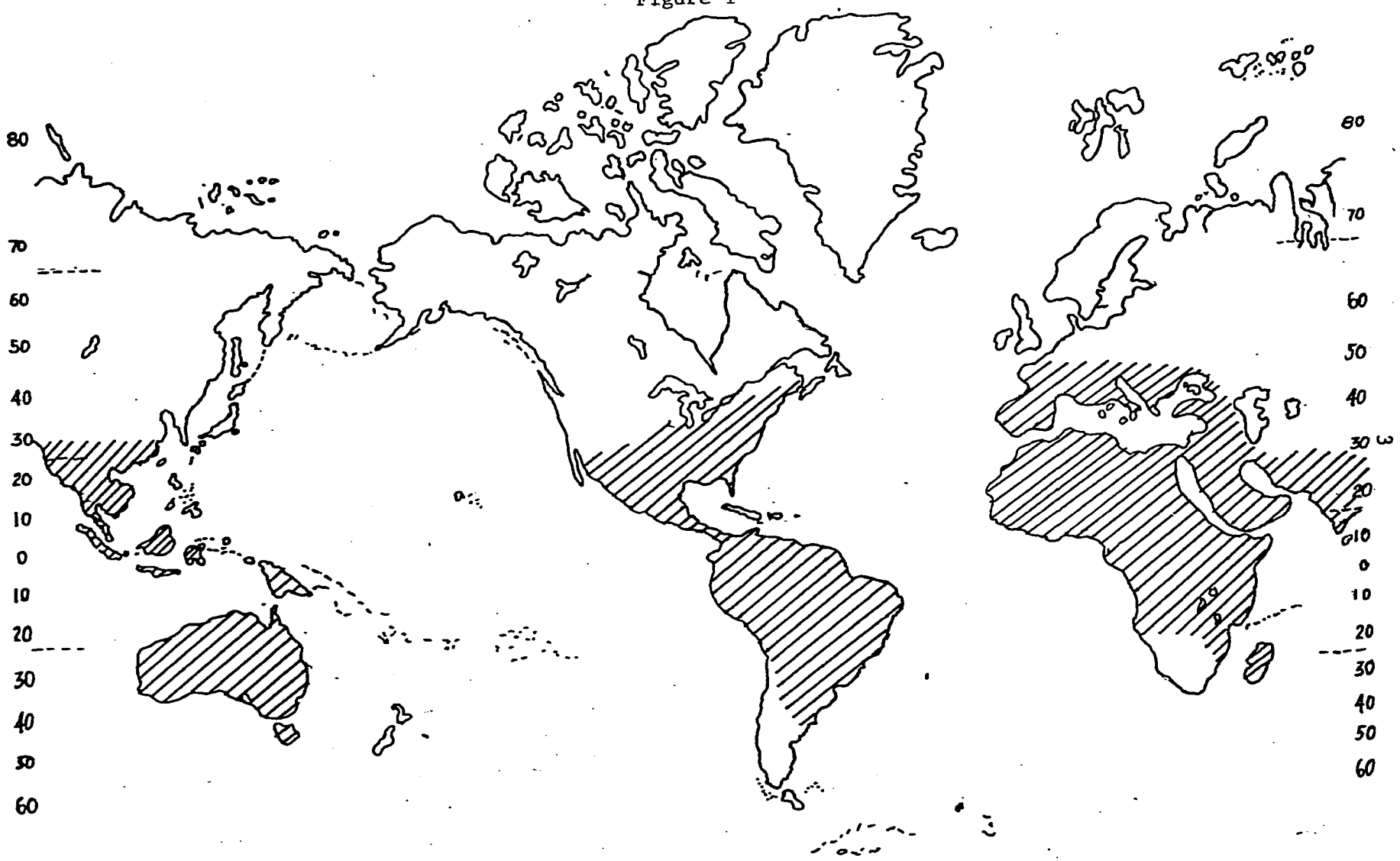
Aedes aegypti is the type species of the subgenus Stegomyia Theobald, 1901, of the genus Aedes Meigen, 1818, as amended by Edwards in 1932 to include some fourteen previously erected genera and about 400 species (Christopher, 1960). Although Aedes aegypti is not the type for the genus Aedes, this being Aedes cinereus Mg., it is representative in many respects of this large section of the Culicini, which differs from another large section, the genus Culex and allied genera, in being composed largely of dark and often highly ornamented species.

Aedes aegypti is one of the few mosquitoes that, with the aid of man, is distributed around the entire globe between latitudes 35 South and 45 North (Figure I). These limits of distribution appear to be related to temperature. This relation, however, is not a simple one. The northern boundary in America corresponds to a January isotherm of 35°F and a July isotherm of 75°F. The July isotherm includes parts of the British Isles which are well north of the limit and where only one doubtful occurrence of the species has ever been recorded.

The presence of Aedes aegypti throughout the southern half of the United States, Central America, West Indies and parts of Brazil was sufficient at one time to suggest that this was the ancestral home (Christophers, 1960). Dyar (1928) opposed this view basing his idea on the fact that no closely related species exist on the American continent, but many such relationships exist in the Old World, especially in Africa. Not only have numerous species of the subgenus Stegomyia been recorded from the Ethiopian Region (some twenty-seven), the next richest area being the Oriental with thirty, but there are within this region some very closely related forms. Furthermore, absence of the species from much of the forest area of Brazil is in marked contrast to the condition in Africa where it has been found breeding in forests independent of man (Christophers, 1960).

Since the ancestral home of Aedes aegypti appears to be Africa, as proposed by Dyar (1928), it seems reasonable to assume that the diffusion of this species to the subtropics and temperate regions of other regions of the world involved an increase in its ability to withstand a wide range of temperatures. Thus different strains of Aedes aegypti must vary

Figure 1



Distribution of Aedes aegypti

in their ability to withstand cold temperatures. Much evidence points to glycerol having an important role in enabling insects to withstand cold temperatures.

This investigation was conducted to ascertain whether selection could be a factor in the development of cold tolerant from non-tolerant strains of Aedes aegypti.

Knight (1922) demonstrated a marked periodicity in the freezing point of the pentatomid, Perillus bioculatus. In the fall this bug gained hardiness and in the spring lost it. Knight considered that the change in hardiness was colloidal in nature. Repeated freezing of the bug brought the freezing and under-cooling points nearer and nearer together and melting of gelatin and other colloids where the past history influences present behavior.

Sacharov (1930) analyzed the freezable water and fat content of certain cold-resistant and non cold-resistant insects and found the amount of freezable water to be lower and the fat content to be higher in resistant insects. Thus, cold-hardiness seems to depend upon the low water-fat ratio.

Kozhantshikov (1938) indicated that cold-hardiness in insects depends upon the physiological state of the organism, the most resistant being in the diapause phases (prepupae of Croesus septentrionalis, eggs of Lymantria dispar, and pupae of Acronyctinae). Not so hardy were the caterpillars of the tent moth, Lasiocampa quircus, and the prepupa of the cutworm, Agrotis segetum, which were stopped in their development by cold temperatures. Growing caterpillars of the webworms, Loxostege sticticalis and Agrotis segetum are practically non-hardy. The

difference in the cold-hardiness of these groups depends upon the specificity of their cellular respiration. Growing insects show in their cellular respiration the prevalence of the usual oxydases (oxidases) characteristic of all aerobic organisms. In cold-hardy insects cellular respiration is closely connected with anoxypiotic (anaerobic) processes caused by dehydrases (dehydrogenases); their activity is not bound to the living elements of the cells but is closely connected with the presence of unsaturated fatty acids peculiar to insects. Cold-hardiness grows with the increase of the percentage of thermostable respiration.

Rozeboom (1941) indicated that Aedes aegypti was fairly common in Oklahoma and in late summer became a pest in the Stillwater area. To determine the effects of cold on its eggs, three batches were placed in a bottle containing some sand and subjected to winter temperatures in Stillwater, Oklahoma. The following April the eggs were immersed in water in which they hatched and subsequently developed into adults which produced viable eggs. The fact that Aedes aegypti is a domestic mosquito, breeding in artificial containers in and around houses and barns, probably accounts for its residence in the Stillwater area.

In the species and stages of insects studied by Ditman et al. (1943) there was a significant correlation between the capacity for cold resistance and low water content. In the hibernating stage the water content and the temperature to which they were resistant were both lower than in the active stage of the insects.

Salt (1950) developed the theory of crystal nucleus formation and experimental work has produced evidence that, for supercooled water within the limits found in nature (say above -50°C), the greater the supercooling

the more chance there is that a crystal nucleus will form. Time automatically becomes a factor in this process because the chances of such an event happening are related to time. The cold-resistant larvae of the wheat stem sawfly, Cephus cinctus, will serve to illustrate. If the larva is cooled from +20° to -30°C within one-half hour or less, the supercooling point may be -30°C. The same insect placed at a constant temperature of -25°C would probably freeze only after a few hours or days; at -20°C only after a few weeks; and at -15°C only after several months. The time required for a crystal nucleus to form in such a specimen would therefore be a matter of seconds at -30°C, hours or days at -25°C, weeks at -20°C, and months at -15°C. A less cold-hardy specimen would require correspondingly less time to freeze at each temperature except -30°C, at which it could not remain in an unfrozen state.

Ramsay (1951) found the hemolymph of Aedes aegypti larvae, starved in distilled water since the third instar, to have a freezing point of 2.550°C. (The freezing point of distilled water is 2.990°C.)

Lovelock (1953) showed that if glycerol is prevented from entering red blood cells of man by treatment with copper ions before freezing, they are no longer protected against damage. Furthermore, damage occurs at a steadily decreasing rate as the temperature falls and becomes very slow at temperatures below -35°C. Glycerol undoubtedly modifies the shape of the ice crystals formed in its presence, so that its addition to a solution considerably decreases the volume of ice formed on freezing at any given temperature. It is thus understandable that glycerol, because of its effects on crystal formation to these erythrocytes, greatly reduces mechanical freezing injury and therefore probably does the same in cells

of insects.

Investigating the influence of food on cold-hardiness of insects, Salt (1953) found that feeding larvae of the cutworm, Agrotis orthogonia, fed on wheat sprouts, were much less cold-hardy than non-feeding premolt and freshly molted larvae. Feeding larvae of the Mediterranean flour moth, Ephestia kuhniella, fed on whole-wheat flour, itself unfreezable, were much less cold-hardy than premolt and freshly molted larvae.

Salt (1955) stated that fresh insect tissues usually have freezing points between 0°C and -3°C, but they readily supercool to lower temperatures. In fact, it is very difficult to induce freezing in an unharmed insect without supercooling it a few degrees. Supercooling is the tendency of the body fluids to remain liquid at temperatures below their normal freezing point.

Salt (1956a) pointed out that water loss, regardless of method, tends to increase osmotic pressure and that osmotic pressure determines the freezing point and affects the supercooling point proportionately. The other environmental factor commonly suspected of producing cold-hardiness is low temperature. This ingredient does not directly produce changes in cold-hardiness, but may do so indirectly through its influence on a variety of metabolic and other processes, some of which are accompanied by cold-hardiness. This is always the role of temperature; it controls the rate of the reaction and therefore its limits.

Salt (1956b) emphasized the effects of freezing in the insect's body on its survival. Many insects that are susceptible to freezing can live at temperatures down to about -5°C if the exposure is very short. Even at -5°C, 65 to 70% of the body moisture is crystallized. Further ice

formation is progressively more lethal.

Salt (1956c) showed that species of insects differ greatly in their response to chilling: eggs of the two-striped grasshopper, Melanoplus bivittatus, and larvae of the wheat stem sawfly, Cephus cinctus, did not cold-harden at $0^{\circ}C$ or $5^{\circ}C$. Salt concluded that chilling per se was not responsible for cold-hardening. Rather, it appeared that some metabolic process incidentally increasing cold-hardiness while others did not.

In the course of biochemical studies on the embryonic diapause of the Bombyx silkworm, Chino (1957) discovered that the glycogen content of the egg decreases markedly at the onset of diapause and reaches the lowest level at about thirty days after oviposition. When diapause is broken by cold treatment, glycogen content increases progressively even at low temperatures and regains the initial level almost completely. During the diapause period the glycogen is not utilized as an energy source but is largely converted into two kinds of polyhydric alcohols, namely sorbitol and glycerol.

Salt (1957) found that all except one of several freeze-susceptible insects investigated contained little if any glycerol. These were larvae, pupae and adults of the blow fly, Phormia regina, Anagasta kuhniella, the flour beetle, Tenebrio molitor; and the eggs and nymphs of Melanoplus bivittatus; nymphs of the lesser migratory grasshopper, Melanoplus mexicanus; larvae of Cephus cinctus; pupae of the cabbage maggot, Hylemya brassicae; adults of the saw-toothed grain beetle, Oryzaephilus surinamensis; and unidentified aphids from greenhouse alfalfa. The exception was Loxostege sticticalis which contained 2 to 4.5% glycerol. This comparatively large amount of glycerol found in

freezing-susceptible L. sticticalis makes it apparent that the simple presence of glycerol in such amounts cannot be held responsible for protection for at least one species of insect against freezing. All of the freezing-susceptible insects tested, except L. sticticalis, offer indirect support by their lack of glycerol.

Salt (1958) found that a concentration of glycerol as great as 5 molal is built up in larvae of Bracon cephi after hibernation begins in the fall and that this is lost in the spring. Glycerol is directly responsible for the cold-hardening of the larvae in two separate ways: (1) by increasing supercooling and (2) by its protecting action in allowing the larvae to survive even if they freeze.

Dubach et al. (1959), investigating the carbohydrates of insects by chromatographic separation, found that the macerated tissue of the winter dormant larvae of the wood-boring beetle, Melandrya striata, taken from felled wood of the peach-leaved willow, Salix amygdaloides Anderss., contained a high glycerol content. This observation indicated that glycerol might act as an anti-freeze, a view supported by the observation that the larvae and adults of these insects did not contain glycerol during the summer. The dormant black carpenter ants, Camponotus pennsilvanicus pennsilvanicus, and their eggs found in Minnesota were shown to contain, in winter time, about 10% glycerol by weight. The same species of ant in an active state obtained in November, 1958 from Maryland contained glucose and fructose but no glycerol. That the glycerol probably plays a major part in the winter-hardiness of this species of carpenter ant is indicated by its disappearance from the Minnesota ants in about three days when they were brought out of their

state of dormancy by being allowed to slowly attain room temperature (20° to 25°C). The dormant larvae of the European corn-borer, Pyrausta nubilalis, have also been shown to contain glycerol. Although glycerol may well play a major part in the winter-hardiness of insects, it is evidently not the only agent which enables them to survive freezing temperatures, for it was also found that the larvae of the wood-boring beetle, Parandra brunnea, and those of the hermit flower beetle, Osmoderma eremicola, did not contain glycerol.

Wyatt and Meyer (1959) found glycerol in the pupae of the silk moth, Hylaophora cecropia. Glycerol appears at about the time of pupation, gradually increases during diapause, and rapidly disappears when this stage ends and the adult develops.

Atwal (1960) demonstrated that regardless of the temperature and duration of conditioning, Anagasta (Ephestia) kuhniella pupal mortality due to subzero exposure increased as the age increased. This was also the pattern exhibited by the adults.

Salt (1961) pointed out that the apparent low supercooling point of water is -40°C, which is attainable with very small amounts of extremely pure water cooled fairly rapidly. In mixed aqueous systems, on the other hand, it is possible to increase supercooling by inhibiting nucleation. This occurs when molecular travel is restricted by hydrogen bonding as in viscous syrups or gels. The supercooling of larvae of Bracon cephi (Gahan) ranged from 26° to 36°C and the amount varied in direct proportion to the solute concentration, most of which was glycerol. Glycerol has a dual effect: (1) lowering the freezing point and (2) increasing the supercooling at least an equivalent amount. A larva of

Bracon cephi which supercools to -45°C will have a freezing point of -15°C, so that the amount of supercooling is only about 30°C. These are freeze-tolerant insects. In contrast, freezing-susceptible insects have freezing points only a degree or two below 0°C.

CHAPTER II

MATERIALS AND METHODS

In order to be assured a continuous supply of eggs, several stock cages of adult mosquitoes were maintained. The original stocks of eggs were obtained from Dr. G. B. Craig, Department of Biology, University of Notre Dame, Notre Dame, Indiana. These eggs represented four strains of Aedes aegypti which are classified according to Mattingly in 1957 as follows (Mimeographed letter from Dr. Craig):

I. Geographic.

A. Type form, Aedes aegypti aegypti

1. Cucuta/1961: from Cucuta, Columbia, South America.

2. Kuala: from Kuala Lumpur, Malaya.

3. Rock: Rockefeller Institute Strain.

B. Aedes aegypti formosus (a subspecies from Equatorial Africa).

4. Ssisa: from eggs deposited in bamboo pots, Ssisa, Uganda.

A temperature of 32°C and an average relative humidity of 75% were maintained for all stock mosquitoes.

Adult stocks were maintained in screen cages that were one foot square. Pieces of cotton were placed in small petri dishes over which a 10% sucrose solution was poured to serve as food for the adult males. The adult females were fed every other day on a guinea pig from whose

abdomen and back the hair had been clipped.

Since females of Aedes aegypti oviposit above the surface of the water instead of directly in the water as do some mosquitoes, strips of paper toweling, approximately 2 x 12 inches, the ends of which were brought together and stapled, were used to collect the eggs. These cylinders were easily inserted and removed from the finger bowls.

MacGregor (1916) has experimentally shown that in order for eggs to be resistant to dessication, they must be in contact with water for a period of time. Fresh eggs, upon removal from water long enough for egg shell collapsing to occur, fail to develop when returned to water. The ideal range of time was reported to be between 48 and 72 hours. According to MacGregor, this contact with water either allows for development of the embryo and its membranes or for some change in the egg shell which will provide protection against dessication.

A drying period of 96 hours maximum, as observed by Johnson (1937), yielded a faster and more complete hatch. Therefore, the eggs were collected and placed in a covered container to "age" for three or four days. Care was taken not to dry the eggs too rapidly, since rapid dessication would kill the embryos. After proper drying, the eggs were flooded and the resulting larvae were used for the next experiment. After flooding the eggs, a small amount of Purina Dog Chow was added to initiate bacterial action (Rozeboom, 1934).

Experiments were started in the morning in order to determine the hatching time. After the eggs hatched, the first instar larvae were transferred to the appropriate temperature cabinet and the experiment was started. When pupae appeared, nylon netting was taped over the

top of each container to prevent the adults from escaping.

The number of eggs for each experiment was estimated by counting the eggs of representative samples. The paper toweling provided approximately 20 square inches on which the females could oviposit. The papers were graded excellent, very good, good, fair and poor by containing approximately 100, 50, 30, 20 and 10 eggs per square inch, respectively.

Mortality was determined by counting the individuals that lived in the case of high mortality, or by counting the dead in the case of low mortality.

Experiments were conducted in "Precision" temperature cabinets (modified commercial refrigerators) which were capable of maintaining constant temperatures both above and below room temperature. Four of these cabinets were employed, each being set for a different temperature. Temperatures of $5^{o}C$, $10^{o}C$, $15^{o}C$ and $20^{o}C$ were used for experimental purposes. Each strain of mosquito was subjected to all four temperatures. Each experiment was repeated four times and the data represented an average of the four trials. Upon completion of the life cycle, adults were transferred to cages and maintained at $32^{o}C$ to serve as stock colonies for succeeding experiments at the same temperature from which they had been removed.

To determine the effects of repeated exposures to low temperatures with regard to increased tolerance, vapor pressure determinations were made and quantitative tests were conducted for free amino acids and glycerol.

Osmolality and freezing point determinations were made utilizing

the Mechrolab Vapor Pressure Osmometer, Model 301A. The operating principle is that of vapor pressure lowering. A solution of any given solvent always has a lower vapor pressure than the pure solvent. In the Model 301A, a drop of solution and a drop of solvent are suspended, side by side, in a closed chamber saturated with solution vapor. Because of the difference in vapor pressure of the two drops, a differential mass transfer will occur between the two drops and the solvent vapor phase resulting in a greater condensation of (or lower evaporation from) the solution drop than from the solvent drop. This transfer will cause a temperature difference between the two drops (because of the heat of vaporization) which is proportional to the vapor pressure lowering, and hence to the solute concentration. Since the shift is a colligative effect dependent solely upon the number of dissolved molecules and independent of their chemical characteristics, the instrument may be calibrated with a concentration series of a known solute. The calibration curve was constructed by determining the freezing point depressions of known concentrations of sucrose, and the unknown solvents were read directly from the curve (Instructional Manual for the Mechrolab Vapor Pressure Osmometer, Model 301A).

Micks and Ellis (1951) prepared ethanol extracts of Aedes aegypti adults which were subsequently analyzed for free amino acids. The amino acids or amino acid complexes identified were glutamic acid, taurine, serine-glycine, threonine-lysine, tyrosine-alanine-beta-alanine, valine, arginine, tryptophan, leucine, methionine, histidine, and proline.

During the current investigation the free amino acids were separated by two-dimensional paper chromatography after the method utilized in

Zoology 214 (Comparative Physiology) at the University of Oklahoma. This method involved descending chromatography and a two-solvent system. The first solvent was n-butanol-acetic acid-water (4:1:5), and the second solvent was phenol-water (4:1). The fourth instar larvae of all four strains from all four experimental temperatures were utilized. They were mashed directly on Whatman No. 1 paper and placed in the solvent systems at room temperature (26°C). The developing reagent was 0.5% ninhydrin in acetone.

Tests for glycerol were conducted according to the method of Wyatt as modified by Salt (1957). The solvent was n-butanol-acetic acid-water (4:1:5). The developing spray reagent was that described by Lemieux and Bauer (1954) which consists of four parts 2% aqueous sodium metaperiodate and one part 1% potassium permanganate in 2% aqueous sodium carbonate solution. Larvae were homogenized in 60% ethanol. This was centrifuged, the supernatant portion evaporated to dryness, and taken up in one milliter of water. Chromatograms were run on Whatman No. 1 paper at room temperature (26°C).

CHAPTER III

RESULTS AND DISCUSSION

Previous investigations conducted on Aedes aegypti provided an a priori basis for an hypothesis that cold-tolerance in this species might be increased by selection. Sixteen degrees Centrigrade was found to be the low threshold of development for this species, but some individuals would survive various exposures to much lower temperatures. Assuming these survivors to be the most cold-tolerant, it seemed reasonable to think that by exposing the progeny of these survivors to low temperatures for several successive generations, this tolerance could be increased.

For the current investigations adult and larval controls were maintained at a temperature of 32°C which was as close to the optimal of 36°C as available constant temperature facilities would permit. At this temperature all strains exhibited very active larvae and pupae. Development was rapid, all developmental stages being rather small. The time required for completion of the life cycle varied only slightly among the four strains (Table I). It is interesting to note that while Cucuta, Kuala and Ssisa are from the same general latitude, Kuala completed the life cycle in less time.

Development of all developmental stages was slowed down at a

TABLE I

Effects of Exposure to 32°C. (Controls)

Strain	Time Required to Complete Life Cycle	Mortality (per cent)
Cucuta	11.3 days	10
Kuala	9.3 days	10
Rock	9.6 days	10
Ssisa	11.3 days	10

temperature of 20°C resulting in individuals which were 30-40% larger than the controls. Speed of movement was reduced, but all forms were reasonably active. At this temperature there was remarkable uniformity in the time required for completion of the life cycle in the four strains from generation to generation (Table II). Cucuta and Ssisa required slightly less time than Kuala and Rock. Mortality, however, was approximately 5% for all stages. Egg production remained a constant 30 eggs per square inch from generation to generation.

The rate of development was greatly inhibited by an exposure to 15°C (Table III), resulting in the largest developmental stages of all experimental temperatures. The first 48-72 hours proved to be the most critical period, as most of the larval deaths occurred during it. Fifteen degrees Centrigrade proved to be the lowest temperature which permits proper functioning of all metabolic processes, as evidenced by the fact that below this temperature Aedes aegypti cannot complete development. Low temperatures act indirectly to control the rate and therefore the limits of reactions (metabolic processes), some of which may result in increased cold-tolerance. Movements were greatly slowed down but the larvae and pupae were by no means immobilized. Mortality remained approximately 50% in all generations but egg production steadily declined.

An exposure period of three days to 10°C was maximum for all four strains as a longer exposure resulted in 100% mortality. Within an hour after subjection to this temperature all larvae were immobilized, but the survivors soon revived after removal to room temperature (26°C). The time required for completion of the life cycle as well as mortality

TABLE II

Effects of Exposure to 20°C.

Strain	Generation	Life cycle Completed	Time Required to Complete life cycle	Mortality (per cent)
Cucuta	F_1	Yes	20.0 days	5
	F_2	Yes	17.0 days	5
	F_3	Yes	21.1 days	5
	F_4	Yes	24.8 days	5
	F_5	Yes	21.6 days	5
Kuala	F_1	Yes	21.5 days	5
	F_2	Yes	22.2 days	5
	F_3	Yes	24.8 days	5
	F_4	Yes	26.4 days	5
	F_5	Yes	23.4 days	5
Rock	F_1	Yes	19.3 days	5
	F_2	Yes	24.0 days	5
	F_3	Yes	26.3 days	5
	F_4	Yes	25.8 days	5
	F_5	Yes	24.3 days	5
Ssisa	F_1	Yes	21.5 days	5
	F_2	Yes	15.2 days	5
	F_3	Yes	24.0 days	5
	F_4	Yes	21.8 days	5
	F_5	Yes	19.0 days	5

TABLE III

Effects of Exposure to 15°C.

Strain	Generation	Life Cycle Completed	Time Required to Complete Life Cycle	Mortality (per cent)
Cucuta	F_1	Yes	45.3 days	50
	F_2	Yes	38.0 days	50
	F_3	Yes	45.3 days	50
	F_4	Yes	47.6 days	50
Kuala	F_1	Yes	43.6 days	50
	F_2	Yes	40.0 days	50
	F_3	Yes	49.0 days	50
	F_4	Yes	49.7 days	50
Rock	F_1	Yes	45.6 days	50
	F_2	Yes	47.5 days	50
	F_3	Yes	43.1 days	50
	F_4	Yes	52.0 days	50
Ssisa	F_1	Yes	41.3 days	50
	F_2	Yes	41.5 days	50
	F_3	Yes	44.5 days	50
	F_4	Yes	40.7 days	50

varied from generation to generation (Table IV). Egg production declined from 30 to 10 eggs per square inch. The adults were maintained at a temperature of 32°C.

An exposure of two days to 5°C produced an average mortality of 98% which remained relatively unchanged from generation to generation. After a few minutes exposure the larvae were completely immobilized but the ones that survived soon revived after removal to room temperature (26°C). The time required to complete the life cycle varied among the four strains from generation to generation (Table V). Egg production declined from 30 to 10 eggs per square inch.

Data from the exposure of larvae to low temperatures show no significant change in the time required for completion of the life cycle in any of the four strains of Aedes aegypti. Each generation was essentially a new experiment, as no survival value was derived from the previous generation's exposure. An exposure of 20°C resulted in a constant low mortality in all strains through all generations with all strains requiring about the same time for completion of the life cycle. At this temperature egg production remained at 30 eggs per square inch.

The time required for completion of the life cycle for the four strains at 15°C was greatly increased but remained relatively constant from generation to generation. The greatest changes occurred at 10°C and 5°C where the previous generation's exposure appeared to influence the present generation. This was manifested by an increased mortality in all four strains from generation to generation to a point of virtual extinction.

Repeated exposure to low temperatures (15°C, 10°C and 5°C) had the

TABLE IV

Effects of Exposure to 10°C.

Strain	Generation	Life Cycle Completed	Time Required to Complete Life Cycle	Mortality (per cent)
Cucuta	F_1	Yes	17.0 days	50
	F_2	Yes	22.7 days	70
	F_3	Yes	20.6 days	90
	F_4	Yes	17.0 days	90
	F_5	Yes	17.3 days	90
Kuala	F_1	Yes	18.2 days	50
	F_2	Yes	21.6 days	70
	F_3	Yes	23.0 days	85
	F_4	Yes	20.2 days	85
	F_5	Yes	18.7 days	90
Rock	F_1	Yes	17.0 days	50
	F_2	Yes	22.0 days	65
	F_3	Yes	21.6 days	90
	F_4	Yes	16.4 days	90
	F_5	Yes	15.5 days	95
Ssisa	F_1	Yes	16.0 days	65
	F_2	Yes	19.5 days	80
	F_3	Yes	19.6 days	95
	F_4	Yes	18.6 days	98
	F_5	Yes	19.0 days	98

TABLE V

Effects of Exposure to 5°C.

Strain	Generation	Life Cycle Completed	Time Required to Complete Life Cycle	Mortality (per cent)
Cucuta	F_1	Yes	14.3 days	98
	F_2	Yes	18.5 days	98
	F_3	Yes	19.5 days	98
	F_4	Yes	18.0 days	98
	F_5	Yes	17.2 days	98
Kuala	F_1	Yes	15.0 days	98
	F_2	Yes	18.5 days	98
	F_3	Yes	21.0 days	98
	F_4	Yes	15.7 days	98
	F_5	Yes	15.0 days	98
Rock	F_1	Yes	14.0 days	98
	F_2	Yes	18.0 days	98
	F_3	Yes	18.6 days	98
	F_4	Yes	18.3 days	98
	F_5	Yes	17.1 days	98
Ssisa	F_1	Yes	14.0 days	98
	F_2	Yes	16.0 days	98
	F_3	Yes	19.0 days	98
	F_4	Yes	17.1 days	98
	F_5	Yes	18.0 days	98

general effect of reducing fecundity in all strains of Aedes aegypti. Exceptions to this reduced fecundity were the F_2 generations of all strains at 10°C and 5°C. The most dramatic exception was Kuala at 5°C, where egg production went from approximately 30 eggs per square inch to 100 eggs per square inch. This increase was temporary, however, as subsequent generations of this and other strains were characterized by a general decline in fecundity.

The average freezing point of the four strains of Aedes aegypti used was -0.237°C, which corresponds quite well to a freezing point of 2.550°C as demonstrated by Ramsay (1951) in Aedes aegypti which had been starved in distilled water since the third instar.

Osmolality and freezing points were determined comparing fourth instar larvae of the F_5 generation with the fourth instar larvae of stocks or controls (F_0). This was done for all four strains from all four experimental temperatures (Table VI). It must be stated that these data are based on only one sample from each strain. This was necessary because of the large numbers of larvae needed to provide the two or three drops of sample solvent required for determinations.

The osmotic concentration is usually expressed in osmoles, which is the total number of moles of particles per liter of solvent. This concentration is due to the colligative properties of molecules. From any one of the colligative properties the others can be calculated; the higher the concentration of solute, the greater are the osmotic pressure, the lowering of vapor pressure, the elevation of the boiling point and the depression of the freezing point of a solution. A one osmolal aqueous solution freezes at -1.86°C. The freezing point can

TABLE VI

Osmolality and Freezing Points.

R	Osmoles	F.P. in C.	Strain	Experimental Temperature (C).
15.03	0.120	-0.223	Cucuta	5
15.93	0.125	-0.232	Kuala	5
16.11	0.128	-0.238	Rock	5
16.00	0.127	-0.236	Ssisa	5
15.66	0.123	-0.229	Cucuta	10
16.00	0.127	-0.236	Kuala	10
17.00	0.139	-0.259	Rock	10
16.89	0.138	-0.257	Ssisa	10
14.05	0.105	-0.201	Cucuta	15
16.14	0.128	-0.238	Kuala	15
17.36	0.140	-0.260	Rock	15
17.16	0.139	-0.259	Ssisa	15
14.97	0.115	-0.214	Cucuta	20
15.36	0.118	-0.219	Kuala	20
16.33	0.129	-0.240	Rock	20
16.55	0.132	-0.246	Ssisa	20
15.31	0.118	-0.219	Cucuta	32 (Controls)
15.10	0.117	-0.217	Kuala	32 (Controls)
15.30	0.118	-0.219	Rock	32 (Controls)
16.30	0.129	-0.240	Ssisa	32 (Controls)

thus be obtained by the formula:

$$f.p. = -1.86 \times \text{Osmolality}$$

As pointed out by various investigators (Ditman, 1943; Salt, 1955, 1956a), a change in the osmotic pressure results in a proportional change in the freezing and supercooling points. That there was no change in the cold-tolerance is evidenced by the fact that there was no change in the osmotic pressure in any of the four strains of Aedes aegypti.

Most of the amino acids identified by Micks and Ellis (1951) from adults of Aedes aegypti were identified during the present investigation. The distribution of the amino acids, both quantitatively and qualitatively, were essentially identical for all four strains of Aedes aegypti from all experimental temperatures.

The results of glycerol determinations were negative. No glycerol was found in any strain before or after continual exposure to the experimental temperatures.

Recent investigations cited previously have indicated that glycerol has a direct bearing on the insect's ability to withstand low temperatures. Low temperatures, especially freezing temperatures, can kill in a number of ways: (1) protoplasmic organization is disrupted by the formation of ice crystal; (2) metabolism is slowed down; and (3) enzymes differ in their temperature optima, and even above freezing some enzymatic action is lost. The presence of glycerol increases supercooling and protects the insects even if they do freeze. The strains of Aedes aegypti utilized during the present investigation did not have glycerol present in the hemolymph or, if it was present, the amount was so small as to be indeterminable.

My data show that prolonged exposures to low temperatures result in high, unchanging mortality, no significant change in the time required to complete the life cycle, decreased egg production and no change in osmolality. It can therefore be concluded that Aedes aegypti of the strains employed cannot successfully adapt to low temperatures.

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