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Study on *Anopheles stephensi* Var. *mysorensis* - effect of low temperature on tolerance of eggs hatchability

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Abstract- Experiment was designed to find out the effect of low temperature on tolerance of eggs of *Anopheles stephensi* Var mysorensis. Eggs were exposed to various length of time to two low temperature regimes 2.0 ± 0.5 °C and 4.0 ± 0.5 °C to study the relationship between temperature and hatchability (percentage hatching) and hatching time of eggs following their incubation at normal 30.0 ± 0.5 °C.

Key words: *Anopheles stephensi* Var. *mysorensis*, Hatching, Pond water, temperature, Instar, larvae, Mortality, lethal, larval life

INTRODUCTION

The insect *Anopheles stephensi* selected for the present study is an established vector of malaria. Like other insects, mosquitoes, including the species used for experimental studies, possess morphological, physiological and behavioural adaptation for survival and reproduction. It has to face periods of environmental stress for which it develops specific strategies to ward off adverse environmental conditions. Global warming may affect the future pattern of many arthropods borne diseases, yet the relationship between temperature and development has been poorly described for many key vectors. The extent to which the *Anopheles stephensi* are able to tolerate the conditions of stress pertaining to temperature, pH etc. and survived and reproduced has formed the subject of present investigations. *Anopheles stephensi* Liston, 1901 is not a homogenous species. Sweet and Rao (1937)[1] and Sweet *et al.* (1938)[2] classified this species into two races – the type form and Var *mysorensis*. Puri (1949)[3] raised *mysorensis* to the sub specific status but Knight and Stone (1977) have listed it as a synonym. Sweet and Rao (1937,1938)[1,2] distinguished the two forms on the basis of egg characters. Rao (1984)[4] treated *mysorensis* as a variety. Criteria of egg characteristics taking into account their length, breadth and dimension of floats of the species used during the present study tend to suggest that the species collected were *mysorensis.* Hence, this has been followed throughout the text. The type form is predominantly an urban mosquito while *mysorensis* is largely rural in distribution.[5] Contrary to the findings of Knowles and Basu (1934)[6], Ganguli (1935)[7] and Strickland (1936)[8], *Anopheles stephensi* Var *mysorensis* is a very abundant mosquito of Hazaribag and its rural areas. These were collected in large number from the cattle sheds and less commonly from houses. Recent studies by Y. D. Sharma (personal communication) have show that both type form and *mysorensis* are actually the synonym as there is no difference in the ribosomal RNA of the two forms.

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Singh and Raziuddin, (1997)[9] while conducting a survey of anopheline mosquitoes in Hazaribag have recorded the occurrence of a total of 10 species as follows:

1. *Anopheles annularis*
2. *Anopheles culicifacies*
3. *Anopheles subpictus*
4. *Anopheles fluviatilis*
5. *Anopheles stephensi*
6. *Anopheles vagus*
7. *Anopheles varuna*
8. *Anopheles pallidus*
9. *Anopheles maculates*
10. *Anopheles moghulensis*

Of these *Anopheles stephensi, Anopheles culicifacies, Anopheles annularis,* have been identified as vectors of malaria in and around Hazaribag.[9]

Mosquitoes complete their life cycle in aquatic medium and as matter of fact range of temperature variation tends to be less in water than on land and aquatic organisms have a narrower limit of tolerance to temperature than the land animals. This is because the water has several unique thermal properties that combine to minimize temperature changes, thus the range of variation is smaller and change occurs more slowly in water than in air. The most important of these thermal properties of water are:

1. High specific heat.
2. High latent heat of fusion.
3. Highest known latent heat of evaporation and
4. Water has its greatest density at 4°C.

It is, therefore, temperature is very important limiting factor.[10]

Temperature, one of the easiest of environment factors to measure, is also generally responsible for the zonation and stratification of animals in water and land. Extremes of temperatures are avoided and at temperatures approaching the upper lethal temperature, insects become highly active. In a similar manner movement to an area of low temperature has been found to promote a brief burst of activity. This may tend to take the insect out of the unfavourable area, so that it is neither killed by extreme heat nor trapped at temperatures too low for its metabolism to continue efficiently.[11]

The optimum range of temperature is always variable between the species and within the species. However, an insect may tolerate an exposure to fatal temperature during a certain stage of the life cycle. Mosquito's developmental stages are aquatic and as a matter of fact along with certain insects, the aquatic stages tolerate the lower temperature during fall and winter.

As temperature variability is extremely important ecologically, a temperature fluctuating between 10°C to 20°C and averaging 15°C does not necessarily have the same effect on the organisms as a constant temperature of 15°C. It has been found that organisms which are normally subjected to variable temperature in nature (as in most temperate regions) tend to be depressed, inhibited or slowed down by constant temperature. Thus, to give the results of one pioneer study it was found that the eggs and larval or pupal stages of the codling moth developed 7 or 8 per cent faster under condition of variable temperature than under a constant temperature having the same mean. In another experiment grasshopper eggs kept at a variable temperature showed an average acceleration of 38.6 per cent and nymphs an acceleration of 12.0 per cent, over development at comparable constant temperature. It is not certain whether variation itself is responsible for the acceleration effect or whether the higher temperature causes more growth than is balanced by the low temperature. In any event, the stimulating effect of variable temperature, in the temperate zone at least, may be accepted as a well defined ecological principle, and one that might be emphasized, since the tendency has been to conduct experimental work in the laboratory under constant temperature conditions.

Since temperature is the most important of the abiotic factors affecting the life of insects, it was felt necessary to find out answers on the effect of constant and variable temperature conditions on the embryonic and post embryonic development of one of the most important vectors of malaria, *Anopheles stephensi* which is a common species throughout the Jharkhand.

In the present work an attempt has been made to find out the effects of constant and as well as variable temperature regimes on the development of aquatic stages of *Anopheles stephensi.*

Amongst mosquitoes, the species which have been investigated frequently are *Anopheles maculipennis, Anopheles annularis, Anopheles subpictus, Anopheles gambiae, Culex pipens pipens, Culex teralis,* and *Aedes aegypti.*[12] References in connection with a more species, such as *Anopheles subpictus*, *Anopheles gambiae, Culex papien fatigans, Aedes commonis, Aedes taeniorhynchus, Culiseta annulata, Culiseta moritans, Mansonia sp*., and *Psorphora aliata*, are available.[13-15] It is surprising that common Indian anopheline mosquito *Anopheles stephensi*,

primary vector of malaria has not received as much attention as it deserves in Jharkhand on account of its economic importance as a carrier of *Plasmodium* in man and for also being a major vector of malaria in Hazaribag.[9]

A survey of literature reveals that work done on *Anopheles stephensi* relate to seasonal trends in population, time and survivorship[16], taxonomy[2,3], distribution[17-23], adult bionomics[6-8,21,23-30], biting and feeding habits[17,31-34], flight and dispersal[29,35-38], host preference[16,27,34,39-41], swarming and mating[42,43], light preferences[44-46], relation to disease[1,5,16,22-25,27,34,38,47-49] and control[4,41,50,51].

From the survey of literature, it also appears that although a large volume of work has been taken on *Anopheles stephensi* with reference to its taxonomy, bionomics and its relation to malaria, perhaps only limited amount of experimental work is available with reference to some most important abiotic factors viz; temperature and pH of water in which development occurs. Some recent studies on these aspects are under Hoffman and Manisubramanian (2005)[46] investigated the role of light exposure on the final stages of development of *Anopheles stephensi* larvae to pupae and adult mosquitoes. Nath and Lakhotia (1989)[52] studied heat shock response in ovarian nurse cells of *Anopheles stephensi*. Noden *et al.* (1995)[53] have investigated the impact of temperature on early *Plasmodium falciparum* development in *Anopheles stephensi*. Ives and Paskewitz (2005)[41] have tested the effect of vitamin B on attractiveness of *Anopheles stephensi* and found no repellent effect on human subject after vitamin B supplementation. Ansari *et al.* (2005)[51] have tested efficacy of hilmilin, an insect regulator, against *Anopheles stephensi*.

In general, of literature survey shows that although a large amount of work has been carried out on the biology of anophelines, egg development and also development of larvae and pupae of *Anopheles stephensi* Var *mysorensis* in relation to environmental parameters is an understudied aspect of vector biology. Work done on these aspects in other species of *Anopheles* cannot be generalized as because some species of *Anopheles* have been found to be more susceptible to lower and higher temperatures, thus temperature can influence development in a differential manner even when related species are considered.[54] It was therefore, the present work was undertaken.

MATERIAL & METHODS

Anopheline mosquitoes are abundantly found in and around residential localities of Hazaribag town. Although they could be found all the year round, they were plentiful from mid of June to mid of October. *Anopheles stephensi* females were easily identified by its light fawn (grey-brown) body colour and speckling in legs (mainly of the femora and tibiae).

These were collected from their indoor resting habitats viz; houses and cattle-sheds during evening hours. Mainly the blood fed females were collected from resting habitats.

Mosquitoes were collected generally by using a test tube or an aspirator consisting of a glass tube 20 mm in diameter, a rubber tube and a mouth piece. While using the test tube, the mouth of the tube was slowly applied over the mosquito in such a way that when it tended to fly, it fell into the tube. Usually not more than two blood fed mosquitoes were trapped in a single test tube of (1.5 cm). The mouth of the test tube was closed with cotton wool. Every care was taken to avoid damage. (Figure 2.1a and 2.1b)

The collected mosquitoes were brought to the laboratory and kept in a specially designed breeding chamber. The breeding chamber consisted of a steel frame work (2.7 feet x 1.2 feet x 1.2 feet). The top and bottom of the chamber were covered by steel sheet painted ash grey. The four sides of the chamber were covered by a fine white nylon net which prevented the escape of mosquitoes, but allowed free entry of air and also permitted observations from outside. On one side of the net, a hole was made with which one-foot-long tube-like net was sewed. Through this tubular netting hand could be easily entered into the breeding chamber (Figure 2.3). This tubular netting was tightened at its base all the time except when there was a need to enter the hand for releasing mosquitoes. Eggs were collected from rearing cages within one hour after deposition and subjected immediately to the experimental conditions. Counts of larvae were made at hourly intervals throughout the period of hatching and scored. A daily score of hatching with respect to control and experimental lot was made.

Insects in general exhibit an increased rate of growth with a rise in temperature. In particular species the relation of temperature to growth is sufficiently constant to furnish a basis for predicting growth rates at known temperatures. This study was undertaken in order to provide a working basis for predicting the growth rates of *Anopheles stephensi* Var *mysorensis* under the constant and varying temperature conditions.

The effect of constant temperatures on development of eggs, larvae, pupae and adults were performed using the B.O.D incubator. For experiments at temperature below 5°C, a fridge was used. The temperature in the chamber just below the freezing chamber of the fridge was repeatedly noted using a centigrade thermometer and maintained by manipulating the cooling knob of the fridge so that it could be set at desired temperature (2°C- 4°C).

For rearing of larvae pond water was generally used. As recommended by CDC (2007) 2% W/V baker's yeast was added to water for the day of hatching and the third day. Thereafter larvae were provided with fish food obtained from local market. Further, care was taken not to keep the larvae in the crowed condition as it has been shown experimentally that exclusion rate diminishes as density of larvae in the rearing pan increases. High larval density has also been found to be a distorting factor in sex ratio. The guidelines provided for anopheline culture provided by CDC and Malaria Research and Reference Reagent Resource Centre (MR4), USA (in their various publications 2007a, and 2007b)[55,56] have been used while handling the mosquitoes and its different stages.

The findings were obtained and results of successive experiments were recorded and tabled. The experiments were performed in replicates and their mean and standard errors were also calculated with the help of standard deviation. The data were also subjected to regression analysis to get the best fit plot of the concerned parameters with correlation coefficient. Regression analysis and correlation coefficient with experiments are mentioned in appropriate places, tables and figures.

Anopheles stephensi Var *mysorensis* females prefer clean water for oviposition. Under normal controlled conditions the females laid on an average 125 eggs. Eggs are boat-shaped (Figure 4.4). Upper surface of egg as broad as egg body, with slightly narrowed median portion, float touching margin of upper surface, occupying about middle half of the egg; float ridges about 20, rather smooth and regular (Figure 4.4a). Boat-shaped egg measured 478.71 ± 1.56 µm (Mean ± S.E) in length and 201.03 ± 1.40 µm (Mean ± S.E) in breadth, which included width of the air float.

RESULTS

Low temperature tolerance of freshly laid eggs of *Anopheles stephensi* Var *mysorensis* was tested by giving eggs an exposure of various lengths of time to 2.0 ± 0.5 °C and hatchability (percentage hatching) and hatching time of eggs following their incubation at 30.0 ± 0.5 °C were studied. This experiment was done with a view to know what happens to eggs of the mosquitoes, when low temperature regime persists for some time. Do the eggs die or simply their development is arrested and they develop again when suitable environmental conditions return back? Interesting results were found in this investigation, the results of which are shown in table 1 and the corresponding correlation curves giving best fit to the experimental results are presented in Figures 1, 2 & 3 for time of hatching of eggs, total time of hatching and percentage of hatching of eggs respectively versus exposure time.

The eggs die after 20 hours of exposure to 2.0 ± 0.5 °C and these do not hatch. At 2.0 ± 0.5 °C the development of eggs is completely arrested. It is also found that up to 4-hour exposure of eggs to 2.0 ± 0.5°C the time required for hatching is more or less similar to those eggs, which developed at 30°C without exposure to 2.0 ± 0.5°C. However, as the time of exposure of eggs to 2.0 ± 0.5°C increased from 8 to 18 hours, the time required for hatching gradually increased by almost two folds from that required at 30.0 ± 0.5°C and the percentage of hatching decreased from 92.06 % to 11.33 %.

When the eggs were similarly exposed to 4.0 ± 0.5°C for different periods, irrespective of period of exposure to low temperature stress, the time required for hatching of eggs at 30.0 ± 0.5°C is similar to control. This means that the development of eggs remains arrested at 4.0 ± 0.5°C and the eggs exposed to this temperature and then returned back to 30°C behave as freshly laid eggs (Table 2). The results are shown in table 2 and figures 4, 5 and 6. The results of exposure of eggs to 4.0 ± 0.5°C are qualitatively similar to those obtained for exposure to 2.0 ± 0.5°C is so far as percentage of hatching is concerned. But the time of hatching at 30.0 ± 0.5°C remained almost the same showing a very slow rise. These results indicate that the eggs tend to move to dormant states when left exposed to lower temperature below 4.0 ± 0.5°C. Experiments at still lower temperatures could probably have been more interesting, but were not undertaken in this investigation. It will be taken up in future course of our study. The results indeed reveal that the eggs of *Anopheles stephensi* Var *mysorensis* develop a low temperature tolerance by adopting dormant statehood.

Interesting correlations could be established for the time of hatching, total time of hatching and percentage of hatching of eggs with the exposure time.

For exposure at 4.0 ± 0.5 °C, the linear relations of the type

$y = A + Bx$ …….....……..….[6a.0]

were found to give the best fit to the observed data, where,

(i) A = 58.2, B = 0.1518 and r = 0.936 ….....[6a.1] for hatching time versus exposure time

(ii) A = 58.2, B = 1.1518 and r = 0.998 ..…...[6a.2] for total hatching time versus exposure time

(iii) A = 81.995, B = 3.6409 and r = 0.991 …….[6a.3] for percentage of hatching versus exposure time.

Corresponding results for exposure to 2.0 ± 0.5°C, however, did not confirm to the linear relationship. A biquadratic 4th order polynomial relation was found to give best fit to the experimental result:

$y = A + Bx + Cx^2 + Dx^3 + Ex^4$ ……..... [6.0]

where, the values of the parameters A, B, C, D and E along with the correlation coefficient are given in table 2 and figures 4, 5 and 6.

Table 1- Low temperature tolerance of eggs of *Anopheles stephensi* : Hatchability and hatching time of eggs at 30.0 ± 0.5ºC following exposure of freshly laid eggs at 2.0± 0.5 ºC for different periods of time.

Sl. No	Hours at 2.0 ± 0.5 ºC	Time of hatching at 30 ºC (Mean ± S.E) (A)	Total time (hours) for hatching (Mean±S.E) (B)	% hatching (C)	Regression Equation
1.	00	58.3 ± 1.45	58.3	92.06	1. Time of hatching at 30 ºC / Hours at 2.0 ºC (0 -18) : $y = 0.0081x^4 - 0.3226x^3 + 3.9038x^2 - 10.507x + 59.943$, r =0.988*
2.	02	57.6 ± 2.0	59.6	66.00	
3.	04	58.6 ± 3.18	62.6	47.33	
4.	06	73.6 ± 3.18	79.6	37.33	2. Total time (hrs) for hatching / Hours at 2 ºC (0 -18): $y = 0.0083x^4 - 0.3377x^3 + 4.1625x^2 - 10.598x + 60.52$, r=0.992*
5.	08	98.3 ± 6.8	106.3	36.00	
6.	10	106.3 ± 3.93	116.3	35.33	
7.	12	104.0 ± 4.16	126	32.00	3. % Hatching / Hours at 2.0 ºC (0 -18) : $y = 0.0036x^4 - 0.1707x^3 + 2.7627x^2 - 20.085x + 93.399$, r= 0.995*
8.	14	103.6 ± 4.16	117.6	25.33	
9.	16	102.6 ± 1.76	118.6	12.00	
10.	18	105.6 ± 1.76	123.6	11.33	
11.	20	No hatching	-	-	
12.	22	No hatching	-	-	*High degree of correlation
13.	24	No hatching	-	-	

Table 2- Low temperature tolerance of eggs of *Anopheles stephensi* : Hatchability and hatching time of eggs at 30.0 ± 0.5ºC following exposure of freshly laid eggs at 4.0 ± 0.5 ºC for different periods of time

Sl. No	Hours at 4.0 ± 0.5ºC	Time (Hours) for hatching at 30 ºC (A)	Total time (hours) for Hatching (B)	% hatching (C)	Regression equation
1.	0	58.3 ± 1.45	58.3	89	1. Hatching time at 30 ºC / Exposure time (hrs) :
2.	2	58.9 ± 1.45	60.9	74	
3.	4	58.4 ± 1.87	62.4	62	y = 0.1518x + 58.2, r = 0.936*
4.	6	59.1 ± 2.46	65.1	56	
5.	8	59.7 ± 2.89	67.7	52	2. Total time(hrs) for hatching / Exposure time (hr) :
6.	10	58.9 ± 2.11	68.9	46	y = 1.1518x + 58.2 , r = 0.998*
7.	12	60.2 ± 1.27	72.2	39	3. % hatching / Exposure time (hrs) :
8.	14	60.7 ± 2.05	74.7	32	y = -3.6409x + 81.955, r =0 .991*
9.	16	60.3 ± 2.13	76.3	25	
10.	18	60.9 ± 1.55	78.9	18	
11.	20	61.5 ± 1.92	81.5	8	(*High degree of correlation)

Fig. 1- Plot of time of hatching of eggs versus exposure time of *Anopheles annularis* at 30°C following exposure of egg to 2.0°C for different periods of time.

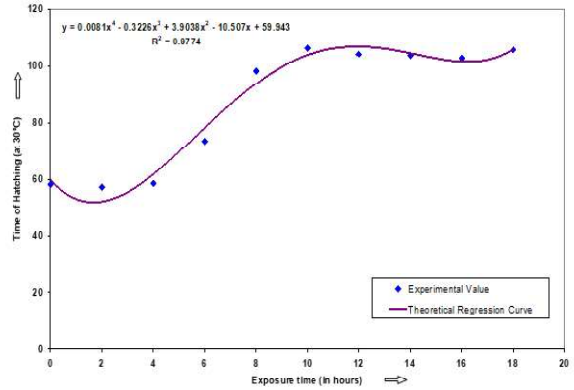


Fig. 2- Plot of time of hatching of eggs versus exposure time of *Anopheles stephensi* Var *mysorensis* at 30°C following exposure of egg to 2.0°C for different periods of time.

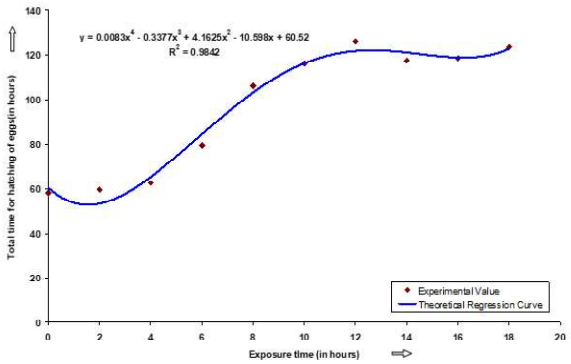


Fig. 3- Plot of percentage of hatching of eggs versus exposure time of *Anopheles stephensi* Var *mysorensis* at 30°C following exposure of egg to 2.0°C for different periods of time.

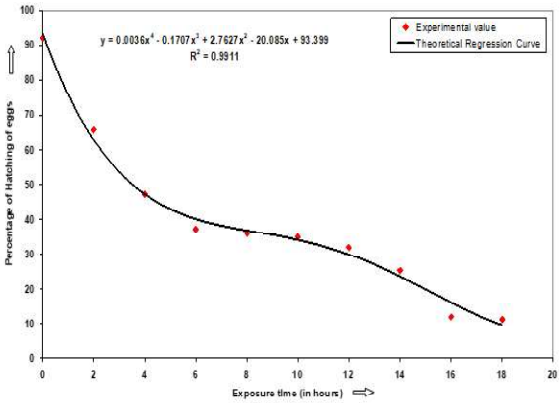


Fig. 4- Plot of time of hatching of eggs versus exposure time of *Anopheles annularis* at 30°C following exposaure of egg to 4.0°C for different periods of time.

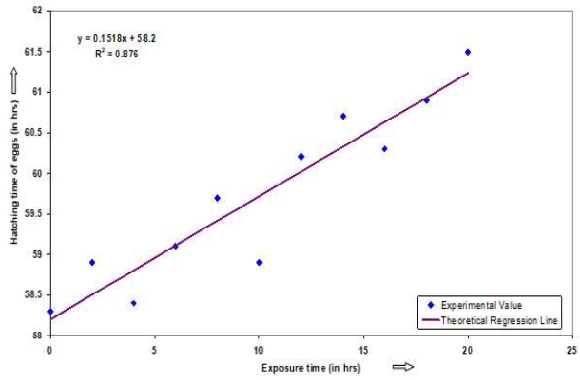


Fig. 5- Plot of time of hatching of eggs versus exposure time of *Anopheles stephensi* Var *mysorensis* at 30°C following exposaure of egg to 4.0°C for different periods of time.

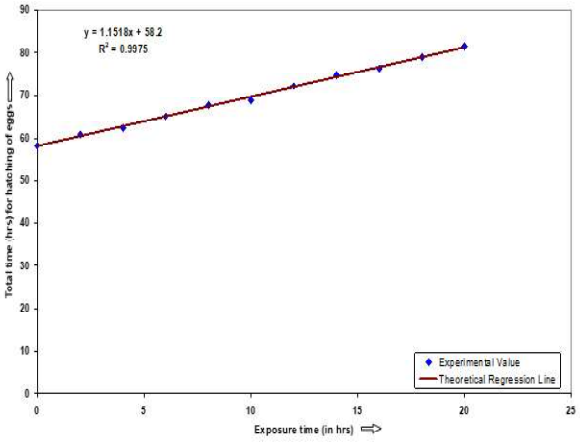
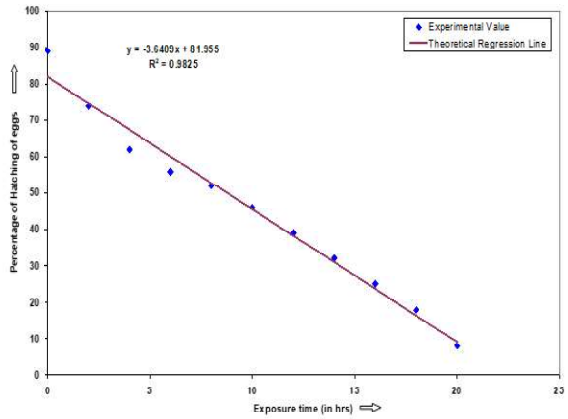


Fig. 6- Plot of percentage of hatching of eggs versus exposure time of *Anopheles stephensi* Var *mysorensis* at 30°C following exposaure of egg to 4.0°C for different periods of time.



DISCUSSION

Different abiotic factors, in particular temperature, moisture and light, air etc has been found to be significant in their influence in insects. Abiotic factor, temperature is not uniform throughout an ecosystem. Further, temperature also differs in different seasons of year. The temperature in the environment very seldom remain fixed and that too for a very short duration. The insects have to bear the effects of constant temperature, which may at time be below or above the optimum temperature range, as well as varying temperature.

Physical and biological factors are important in controlling the actual distribution and abundance of organism in nature. Temperature is one of the most important factors in this regard. It is known that life exists only within a tiny range of temperature. Further, most species and their activities are restricted to an even narrower band of temperatures. In general for an organism the upper limits of temperature are more quickly critical than the lower limit despite the fact that many organisms appear to function more efficiently towards the upper limit of their tolerance ranges.

As far as seasonal prevalence of *Anopheles stephensi* Var *mysorensis* is concerned, the present observations and observations made by earlier authors in different parts of India (Chowdhury, 1936[17]; Rajgopalan *et al.*, 1979[20]) suggest that the seasons of prevalence in most part of the country vary. This obviously appears due mainly to the difference in climatic conditions prevailing in different parts of the country. The observations made during the present study demonstrate that the life cycle of *Anopheles stephensi* Var *mysorensis* are greatly influenced by temperature. A temperature between 25°C to 30°C is most favourable for breeding of the species. Even the selection of the resting places by *Anopheles stephensi* adults are influenced by temperature and humidity of the area.[28] During the present study most of the specimens have been collected from cattle sheds rather than from houses, which shows that the species predominantly bites cattle. These findings are in line with those of Afridi *et al.* (1939)[39] and Bhaskar *et al.* (1946)[27]. On the other hand Nair and Samnotra (1967)[40] have described a very high percentage of *Anopheles stephensi* biting human beings in Broach town in Gujarat.

Results obtained in the present study on the life cycle of the *Anopheles stephensi* Var *mysorensis* largely conform to the findings of other workers on different species of anophelines.[4,15]

In nature constant temperature does not exist, but insect are subject to ever fluctuating temperatures. The effect of fluctuating temperatures actually prevailing in nature is wholly different from that of constant temperatures studied under experimental conditions. It is not also correct to interpret insect behaviour with reference to mean temperatures.

To know the effect of variable temperatures on the development of eggs of *Anopheles stephensi* Var *mysorensis,* experiments were designed to expose freshly laid egg, larvae and pupae to low temperature regimes (2°C and 4°C) for various periods of time and then returned back to optimal temperature (30°C) for further development. Some interesting results have been obtained in this investigation. These results were valuable as a guideline to understand the effect of temperature fluctuations in nature on the development of an insect. The eggs die after 20 hours of exposure to 2°C and accordingly do not hatch. When the eggs were exposed to this temperature for up to 4 hours the hatching time was similar to control ones which were developed at 30°C. But with the increase in exposure time hatching time gradually increased and percentage of hatching decreased. Thus it appears that the eggs develop a low temperature tolerance by adopting dormant statehood. These findings appear contrary to some earlier finding on *Melanopalus maxicanus* and *Papillia japonica*[57] were such experimental fluctuations accelerate egg development. In the present work results obtained for exposure of eggs to 4°C for various lengths of times are largely similar to those described for exposure of eggs to 2°C. At both temperatures a high degree of correlation has been obtained between low temperature exposure time and time of hatching, total time for hatching and percentage of hatching.

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