THE REACTIONS OF MOSQUITO LARVAE TO REGULAR REPETITIONS OF SHADOWS AS STIMULI

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Summary

Two factors are involved in the diving reaction of larvae of Culex fatigans, Wied., a kinetic stimulus which initiates movement and a directional stimulus. The kinetic stimulus may be a water vibration, a change in light intensity, or a mild electric shock. The directional stimulus is normally a combination of light and gravity.

Regular repetitions in change of light intensity as a stimulus result in a diminution in the degree of response. This rate of diminution is affected by the number of larvae present and their age. The decrease in relation to age is probably to be associated with their increasing oxygen requirements.

I. INTRODUCTION

Mosquito larvae, like many other microphagous feeders, have no discrete organs which could be described as being for either offence or defence. The larval stage in the life cycle is essentially for feeding and growth in size. Food material is stored up in the body during this period for use in the non-feeding pupal stage when extensive organic reconstruction takes place preparatory to the emergence of the imago. The pupal stage is similarly defenceless.

In the absence of weapons for self-preservation in these stages, survival is achieved by the development of defensive behaviour patterns. These consist of vigorous swimming movements which may serve to take them away from the source of danger when danger threatens. Both larvae and pupae, being primarily air breathers, must spend a considerable amount of their time attached to the surface film. In this position, larvae can feed and respire. They do, however, make excursions from the surface to the richer feeding grounds in the detritus at the bottom and they can also, with their feeding brushes, scrape attached diatoms and algae from the surfaces of submerged water plants. Pupae occasionally, too, make rapid and vigorous dives after which they float passively back to the surface film. Whilst at the surface, the animals show considerable sensitivity to changes in light intensity and to water vibrations, reacting to such stimuli by diving energetically with violent lashings of the abdomen. Such “crash dives” of larvae can be readily distinguished from the more leisurely descents to the feeding grounds.

There can be little doubt that this diving reaction is a defensive mechanism. It is, as far as can be seen, the only means of defence possessed by the organisms from predators such as birds, or from predaceous fish or larvae of other

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insects which would create vibrations in the water in their approach. Escape movements are not necessarily away from the source of danger but their direction is determined, as will be shown, by the position of the animal in relation to light and gravity. In the present investigation the nature of the response of larvae has been studied and also their reactions to regular repetitions of changes in light intensity.

The defensive reaction, such as has been described, could then be considered as a response to a biologically relevant stimulus. This work was undertaken to determine how long the response would continue to be evinced. In other words, would they become irresponsible to an often repeated warning stimulus?

II. Materials and Methods

_Culex fatigans_ Wied. was used as the experimental animal in all tests described. Wild stocks were used exclusively to ensure that there should be no risk of selection or selective breeding. Egg rafts were collected from an outside aquarium and hatched in the laboratory in filtered water from that aquarium. Larvae used in any one series of tests were hatched from the one egg raft. They were fed on a suspension of "Farex" in water. A few drops of this were added daily to the cultures, the amounts added being determined by the size and rate of growth of the larvae. Sufficient was added to ensure a constant supply of food for the organisms without fouling the water. For newly hatched larvae it was found desirable to make the food very finely particulate by grinding it up with a mortar and pestle but this mode of preparation was unnecessary with larger larvae.

Larvae under test were kept in special vessels made of ⅜ in. sheet "Perspex" cemented with "Perspex" cement. These vessels have a front face 3½ in. square. The sides slope inwards slightly towards the back so that the rear face is 3 in. wide. The depth from front to back is 2 in. Their capacity is about 200 ml. Containers of this form were adopted in preference to circular or rectangular vessels as they facilitated observation of the movements of the contained animals. These vessels containing animals to be tested were placed each in a black-lined recess, 8 in. wide by 5 in. deep by 22 in. high. A 60-watt pearl lamp enclosed in a metal lamp-house having a circular aperture 2 in. in diameter was suspended in the top part of the recess 12 in. above the water. A white card 3 in. by 3 in. was placed immediately behind the jar so that the larvae would be more readily visible. All tests were carried out in a darkroom. Before any series of tests, the animals were subjected to a steady light for at least half an hour.

In experiments involving electrical stimulation an induction coil with an interrupter in circuit was used. The electrodes were pieces of tinfoil the same size as the sides of the jar and stuck on to these. Large electrodes were used so that whatever the position of the larva in the jar, it could not escape the electric field. No absolute measure was made of the current passing but the peak voltage was recorded on a cathode ray oscillograph so that the same
voltage could be used in successive experiments. It was found that if too powerful a shock were given, the larvae continued to make agitated movements for some seconds after the current had been cut off, while too weak a shock caused them to make a few spasmodic movements without losing contact with the surface film. A strength intermediate between these was used. In all cases described the duration of the stimulus was one second.

The reactions of larvae to regular repetitions of change in light intensity were studied by occulting the light above them for one second in each minute. This was done by wiring the lamp through a cut-out relay which was activated by a 4.5 volt battery in circuit with a contact on the second hand of a large electric clock. The relay was placed on a shelf away from the experimental bench. A few "blank" experiments showed that the larvae were irresponsive to the slight noise of the cut-out and also to a shaded red light which illuminated the dial of the clock. For repetitions of the electrical stimulations, the induction coil and its battery were wired up to the contacts on the clock.

III. Nature of the Response

Folger (1946) has studied the diving reactions of mosquito larvae and pupae in response to changes in light intensity and mechanical disturbances. He asserts that the downwards movement of the animals as the result of a stimulation involves a negative phototaxis coupled with a positive geotaxis. He postulated "two stimuli which are joined to bring about the descent. One of these is kinetic and induces movement and the other is directive and determines the direction of movement. A kinetic stimulus is associated with time-rate change of the stimulating agent; a directive stimulus with sustained action by it." A change in light intensity or a mechanical disturbance in the water could act as the kinetic stimulus, the direction of movement then being determined by the position of the animal in relation to the light or gravity source. For instance, when the larvae are stimulated by a change in light intensity or a mechanical shock while the light is above them, they go more or less straight down. When the light is from one side, they tend to go down obliquely away from the light, while, when the light is below them their tendency to dive on stimulation is somewhat reduced.

In the present work, an electric shock was used as the kinetic stimulus. Ten third instar larvae were placed in a jar with the light above them. After the usual half hour allowed for acclimatization five shocks, each of one second's duration, were delivered at intervals of one minute. The movements of the larvae are indicated in Table 1.

The experiment was repeated with the same larvae after a lapse of half an hour during which time they were subjected to a steady light but this time with the only illumination below the jar. In this case 12.5 per cent. went to the bottom during the five stimulations. A reversal of the normal position of the light then has an inhibiting effect on the diving reaction. They were then left in darkness for half an hour. At the end of this time a luminescent screen
was placed behind the jar in place of the white card used in the previous experiment. This made the larvae just visible in silhouette. Previous tests had shown that the larvae were unresponsive to light of this low intensity. Five stimulations were again given at minute intervals and 35.7 per cent. of the larvae went to the bottom. In this experiment gravity alone was the directional stimulus. These tests have been repeated several times in various orders, and with different groups of larvae, for example with light below the jar first and above second and so on, but always with substantially the same results, indicating that these differences are real.

| Table 1 |
|-----------------|-----------------|-----------------|
| **EFFECT OF ELECTRICAL STIMULATION ON LARVAE WITH LIGHT ABOVE** |
| At Surface Before Shock | Left Surface | Went to Bottom |
| 1 | 9 | 9 | 5 |
| 2 | 9 | 9 | 4 |
| 3 | 10 | 10 | 6 |
| 4 | 10 | 10 | 7 |
| 5 | 10 | 10 | 4 |
| Totals | 48 | 48 | 26 |
| **Average 54.2%** |

The influence of gravity working alone is also shown by the following experiment. A group of ten third instar larvae were subjected to an occulting light (one second in each minute) until they were completely insensitive (*vide infra*). This took about an hour. Then for five succeeding minutes an electrical stimulus was delivered simultaneously with the occulting of the light. During five stimulations 30.5 per cent. of the larvae went to the bottom. As the larvae had been made insentitive to change in light intensity as a kinetic stimulus the effective one was purely electrical. Further, as the light was occulted during the period of delivery of the shock, the directional stimulus must have been gravity alone. This figure, 30.5 per cent., compares well with 35.7 per cent. obtained with the electrical stimulus in darkness.

**IV. EFFECT OF REPEATED STIMULATIONS**

When a change in light intensity, i.e. occulting the light, was used as a kinetic stimulus, regular repetition of the stimulus resulted in a fairly rapid diminution in the numbers of larvae which responded to an ultimate condition when all were completely insensitive. The rate of onset of this condition of insensitivity depends on at least two factors, namely the number of larvae present in the group and their age. Many other factors, for example temperature, or amount of food present, will also influence the condition, but these have not as yet been investigated in detail.

The influence of the number of larvae present is illustrated in Figure 1. Here four groups containing respectively 3, 6, 9, and 12 individuals from the
same egg raft and all in their third instar were placed in identical jars. After half an hour of steady illumination, occultation of the light for one second in each minute was started and the movements of the larvae in each jar noted. After ten such occultations, group I (3 larvae) had ceased to respond. Groups II, III, and IV still responded actively but ceased in 34, 50, and 80 minutes respectively. Ordinates in the graph represent the number of larvae which remained at the surface after stimulation, expressed as a percentage of the number at the surface before stimulation. Each point represents the average results of at least five immediately successive stimulations. The data for repetitions of the experiment with different larvae in groups of three gave an approximate $x^2$ of 22.0 on two degrees of freedom for which the probability is less than 0.1 per cent. The figures for replications on both groups of six and nine larvae lead to values of $x^2$ significant beyond 0.1 per cent., showing that the increase in inhibition is real for groups of 3, 6, and 9 larvae. A similar degree of probability has been assumed for larvae in groups of 12. Still larger groups, in which the numbers were too large for accurate counting, continued to show some response after several hours of regular occultations.

It would appear then that there is a slower establishment of inhibition in larger groups than there is in smaller ones. This "mass effect" may be caused by a few larvae in the group being more sensitive to stimulation than others. The mechanical disturbance of their movement might act as a kinetic stimulus for others nearby. The larger the group, the greater would be the possibility of there being several of these "sensitives" present, so the longer the reaction of the group as a whole would continue. It was observed that in group IV (12 larvae) and in other cases where a large number of larvae was tested together, when several were gathered in one corner of the jar, some

![Graph](image)

Fig. 1.—Influence of numbers present on the rate of establishment of inhibition to repeated occultations in third instar *Culex fatigans* larvae. Group I, 3 larvae; Group II, 6 larvae; Group III, 9 larvae; Group IV, 12 larvae.
would respond to the stimulus whereas isolated ones at the other end of the jar remained quiescent. Against this explanation is the also observable fact that when a number of larvae is grouped together, on the surface film, one or two swimming up from below into the middle of the group, even though they nudge several in attaching themselves to the surface film, rarely create a marked disturbance amongst their neighbours. Disturbance alone then is probably not the explanation. It seems then that the additive effect of disturbance and change in light intensity both acting as kinetic stimuli can bring about the continued reaction to stimulation in large numbers.

The effect of age on the reaction is also well marked. In an experiment to test this effect, eighty larvae, newly hatched from a single egg raft, were divided into eight groups of ten. With the exception of the testing to be described, they were kept in as nearly identical position as possible. On the first day group I was subjected to 20 light occultations at intervals of one minute. On the second day, groups I and II were similarly tested, on the third day, groups I, II, and III, and so on. Thus on the eighth day, group I had its eighth trial and group VIII its first. The results are summarized in Figure 2. In this Figure, each heavy vertical line records the number of larvae which remained at the surface on each occultation of the light, expressed as a percentage of those at the surface before the stimulation. A dot on the base line indicates zero inhibition. As the percentage inhibition for the succeeding minute intervals are for the same groups of larvae and are computed from a small number (10 in each group), the results of this experiment are not suitable for a strict statistical analysis. It seems evident from Figure 2, however, that (a) there is little variation between successive groups tested.

![Figure 2](image-url)
The latter is attributable to increasing age, the significance of which is demonstrated by an analysis of the results obtained with each group on its first trial. On the basis of the general nature of the experimental results, viz. that data for replications using other larvae are similar, the figures for the minute intervals taken as independent give \( x^2 = 203.6 \) with six degrees of freedom, the probability being less than 0.1 per cent. Six degrees of freedom only are allowed, as the results of the first two days had to be pooled because so few larvae returned to the surface before the succeeding occulations.

These results suggest that no memory factor is involved from day to day. On the eighth day for instance, group VIII receiving its first series of stimulations showed, if anything, a slightly higher rate of establishment of the inhibition than did group I which on that day received its eighth series. The similarity of the graphs on the same day is noteworthy in all cases. The development of the inhibition can then be reasonably associated with the state of morphological and physiological organization of the larvae on successive days, and also with their increasing oxygen requirements with advancing age.

During the experiment all larvae grew to the fourth instar at closely comparable rates. On the first day all were in the first instar, on the second day in the second. On the third and fourth days the third instar had developed, while by the seventh day all but two individuals in group I were in their fourth instar.

For young larvae, the surface-volume ratio is high and most of the oxygen requirements can be met by surface diffusion of dissolved oxygen through the cuticle. As they increase in size, instar by instar, this ratio decreases and more atmospheric oxygen is required to meet the increasing demand. Hence more time must be spent at the surface by older larvae. In Figure 3 are given the percentages of larvae at the surface before each stimulation on successive days. Using for each of the eight groups the actual and possible number of larvae at the surface before stimulation, taken over the twenty minutes on the first day of the test in each case and taking the data for the minute intervals as independent, as before, gives a 2 x 8 contingency table for which \( x^2 = 807 \), \( n = 7 \) degrees of freedom and \( P < 0.0001 \) showing the significance of the increase in proportion at the surface with time in days. The figures for the third and fourth days and also those for the fifth to eighth days both lead to values of \( x^2 \) significant beyond \( P = 0.001 \), showing that the increase in the proportion at the surface during the third and the fourth instars is real.

No figures have been found in the literature so far indicating the oxygen requirements of successive instars, though Fraenkel and Herford (1939) state that Culex larvae use twice as much oxygen when they have free access to the water surface as they do when they are kept forcibly submerged in oxygenated water. The authors do not, however, give any indication of the age of the larvae experimented upon. If the results indicated in Figure 3 are in any way significant, there should be a marked increase in oxygen consumption, at
least in the third and fourth instars. Preliminary tests on the oxygen requirements of successive instars indicate that such is the case and further that oxygen consumption increases with age during one instar. Further experiments have shown that whereas first and even second instar larvae can survive when kept submerged by a layer of fine netting, third and fourth instar larvae cannot, unless artificial aeration of the water is resorted to. The increased need for oxygen in older larvae keeps them more at the surface. When stimulated, they are less prone to leave it and if forced down by a strong stimulus, they return more rapidly.

![Graph](image)

**Fig. 3.**—Relationship between the age of larvae of *Culex fatigans* and the amount of time spent at the surface.

V. DISCUSSION

Such a cessation in the response to repeated shadows has been noted by several authors working with a variety of animals. Von Uexkull (1897) noted that whereas sea urchins turn their spines towards an appearing shadow they fail to respond after three or four repetitions of the stimulus. Nagel (1896), working with several species of lamellibranchs and gastropods which retract into their shells when a shadow passes over them, states that they are quite unmoved when this has been repeated several times. Yerkes (1906) has recorded conditioning in the annelid worm, *Hydroides dianthus*. These worms retreat rapidly into their tubes when "shadowed" but again, repetition induces inhibition. She found, however, that if a slight tactile stimulus were given immediately after the shadow on several occasions on successive days there was an enhanced response to the shadow alone though after about the eighth day, this response tended to fall off too. Hargitt (1909), also working with the same species, obtained a similar result and showed further that specimens
taken from water of about twenty fathoms or over where shadows were less
dense or non-existent, did not respond at all. Mast (1911, p. 249) reports
from his own observations that mosquito larvae and tubiculous worms soon
fail to respond to ordinary shadows if they are kept in places where shadows
frequently occur.

Miller and Mahaffy (1930) have recorded somewhat similar results in
the reactions of the holostome cercaria, C. hamata. In these larvae a short
rapid swim, generally upwards, alternates with a longer period of quiet sinking.
If a shadow passes over them or if the light intensity is otherwise reduced,
most of them start swimming immediately. These workers found that repeti-
tion of "shadowing" at short time intervals (every one or two seconds) re-
sulted in a rapid inhibition. A lapse of longer periods between "shadowings"
generally resulted in a maintenance of the response, the longer the time interval
between stimuli the greater the probability of there being a response.

Agar (1943) discusses the question of acclimatization to repetitions of a
given stimulus under the heading of "Habituation." Quoting from some of
his own experimental work on garden snails he shows that the phenomenon
depends largely on the strength of the stimulus applied. If this is weak and
innocuous, habituation is generally rapid, whereas with strong or painful
stimuli, habituation does not occur. He argues then that sensory or motor
fatigue could be no more than a small contributory factor as habituation to
mild stimuli is more rapid than it is to stronger stimulations. He quotes the
experiments of the Peckhams (1887) on spiders whose results parallel to some
extent the findings in this paper with mosquito larvae. A spider was stimu-
lated by sounding a tuning fork close to it on its web. It responded by drop-
ning from the web and hanging suspended below it. Shortly, however, it
climbed back up its thread on to the web again. Repetitions of this stimulus
each time it returned resulted in its dropping shorter and shorter distances
each time and returning sooner until after about a dozen trials it ceased to
respond at all. Repetitions of this experiment on successive days brought
about a further diminution in the response until on the sixteenth day there
was no response at all nor was there, with two minor exceptions, for the
succeeding ten days of the experiment.

This habituation over a long period Agar attributes to learning by experi-
ence, while the daily habituation might well have a purely physiological basis.
For the mosquito larvae the explanation is probably different. The spider,
in the course of two or three weeks of the experiment, had not developed
appreciably either morphologically or physiologically whereas the larvae during
the eight days of the experiments described herein had grown from newly
hatched instars to their prepupal condition, involving considerable morpho-
logical and metabolic development. The establishment of the inhibition with
increasing age might well have had a physiological basis, namely, as has been
pointed out above, increased oxygen requirements. Its daily establishment
too is more likely to be physiological than psychological. If it is the latter
there is no indication of "memory" from day to day as any group receiving a particular series of stimulations does not show a greater degree of habituation than do those of the same age which have had fewer or no previous experiences of the stimulations. Further, any suggestion that there is "mass psychology" at work in the experiment summarized in Figure 1 presupposes a social unity or bond in the aggregation. Allee (1931) describes such an aggregation as a "synchronopaedium" and Alverdes (1932) as a "conglobation." Neither term connotes anything more than a chance aggregation brought about by purely external influences, so no social unity in the group can be assumed.

If habituation is physiological, sensory fatigue seems to be more probable than motor fatigue. In the discussion on the experiment described in Figure 1, it was pointed out that slower acclimatization of larger groups was attributable to the additive effects of occultation and the presence of "sensitives," giving a more intense total stimulation than occultation alone. Motor fatigue is unlikely as larvae frequently left the surface voluntarily in between and apparently irrespective of the periodic stimulations and moved about in the water quite actively.

A further explanation of the development of the inhibition is possible. If the diving reaction were evinced to every stimulation emanating from a natural environment, feeding, and thus growth rate, would be seriously impaired. Thus its development to a repetition of mild stimuli can be looked on as a behaviour pattern beneficial to survival. This, Agar (loc. cit., p. 115) describes as "negative learning." The same can be said to be the case with the experiments and observations of Nagel on molluscs and Yerkes on Hydroides, in which, as with mosquito larvae, feeding is a more or less continuous process and in which frequent interruptions would be detrimental.

A few tests have been carried out on the response of mosquito larvae using slight tactile and vibrational disturbances as kinetic stimulants. They were found to develop an inhibition as they did to shadows though perhaps not as quickly. There is an interesting contrast here with *Cercaria hamata*. Miller and Mahaffy (loc. cit.) found that repetitions of tactile stimuli did not bring about an inhibition, at least not until the larvae were fatigued. The explanation here is that *Cercaria*, once it has come in contact with its host, must continue in its activity to ensure penetration of the tissues and thus survival, but the mosquito larva, as pointed out above, develops the inhibition as a beneficial behaviour pattern.

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