THE RELATION BETWEEN THE HATCHING OF
THE EGGS AND THE DEVELOPMENT OF THE
LARVAE OF *STEGOMYIA FASCIATA* (*AÈDES*
CALOPUS), AND THE PRESENCE OF BACTERIA
AND YEASTS.

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INTRODUCTION.

THE clearing action of mosquito larvae in turbid water and their scarcity or absence in clean water, is an old-established popular belief in mosquito ridden districts of both the old and new world. If the presence of certain species of *Anopheles* larvae in clear running water, where they doubtless feed on algae, be excepted, it is probable that this general observation is in the main correct. The fact that numbers of mosquito larvae are frequently present in the small collections of clear water which occur in the cut ends of bamboos, the axils of leaves, or the smaller rock or root pools, is an apparent but not a real contradiction of the correctness of this popular belief, because in these instances one of two possibilities may have occurred. Either the large number of dormant eggs which hatched when rain first filled the receptacle was sufficient to check and control bacterial or yeast development from the start, or, as is more likely in the case of root or rock pools, the turbidity due to bacteria or yeasts had been rapidly cleared as the rapacity of the quickly growing larvae increased beyond the source of nutriment.

Mitchell (1907), referring to *Stegomyia fasciata*, says it is pre-eminently a bacteria-eating "wiggler," presumably on the strength of the known fact that the larvae develop rapidly in sewage-contaminated water.

Howard, Dyar, and Knab (1912) mention bacteria as forming food for mosquito larvae in general terms, stating that the spores of algae, particles of dust, bacteria, protozoa, minute aquatic animals of many different kinds are swallowed. They specifically mention that the

larvae of *Aedes* are largely bottom feeders and may be seen vigorously working over dead leaves and vegetable débris with their mouth organs, apparently removing the fungoid growth which covers them.

Boyce and Lewis (1910) showed that the presence of mosquito larvae added to clean water led to an increase in the number of bacteria. Unfortunately the authors have not put on record the methods they pursued in arriving at this result, consequently we are left in doubt as to whether certain very necessary precautions to avoid fallacies were taken or not. For instance it is not stated in what stage or from what source the larvae they added to the clean water were taken, while, unless these factors were carefully controlled, there would be (as pointed out by the authors of the Carnegie Monograph on the Mosquitoes of North and Central America 1912) grave danger of the introduction of organic matter voided from the larval gut. The fact that an increase of bacteria followed the introduction of the larvae is therefore no valid argument against the popular belief of the clearing action of the mosquito larvae, unless the larvae had been introduced immediately after hatching, or just after a moult; the hatching of the eggs or moulting of the larvae having taken place in comparatively large quantities of clean water.

Bacot (1916) carried out a number of carefully planned experiments in Freetown, Sierra Leone, using newly hatched larvae of *S. fasciata*, with a view to ascertaining the amount and nature of the food consumed by the larvae and whether bacterial development in the breeding pans was a factor in the problem. He found that there existed a very definite relation between the development of bacteria and the growth of the larvae. Subsequently, on his return to England, he discovered, as detailed in a footnote to his report (see Experiments I and II), that water highly charged with organic matter, and swarming with bacteria, exerted a powerful stimulus to hatching on eggs which had not responded to immersion in clean water. The suggestion in his report that the bacteria themselves formed food for the larvae was based on the clearing action they displayed in water, rendered turbid by its enormous bacterial content, in conjunction with the fact that the gut-contents of larvae, taken from this water, showed only a few bacteria per field in contradistinction to the crowded fields displayed when water in which the larvae swam was examined. It being deemed impossible that bacteria could be excluded when the larvae were sweeping small particles of matter into their mouths, the explanation of their scarcity must therefore depend upon their rapid digestion.

A few rough experiments in which masses of bacteria and yeasts scraped from the surface of agar cultures were dropped into water containing larvae, showed that the larvae congregated round and greedily devoured them. Their jaws were evidently used to tear and disrupt the clumps of organisms, while the turbid cloud which formed round the struggling crowd of larvae was rapidly dissipated, presumably by the sweeping action of their mouth brushes.

Before describing the experiments and discussing their results, some preliminary explanations are necessary. For the sake of those readers who have no practical knowledge of mosquito breeding it is necessary to state that the conditions favourable for rearing the adults, though simple, require some nicety of adjustment for success, when the breeding pans are small. The essential points are temperature, the amount of nutriment in relation to the quantity of water, and the number of larvae in relation to both the latter factors. The temperature throughout the experiments under sterile conditions was 75° F. which if not quite high enough to give the speediest passage from egg to adult, is quite favourable for the species.

The amount of nutriment and number of larvae in proportion to the quantity of water were, however, in some of our experiments highly artificial.

In Bacot's report (1916) referred to above, the adjustment of these factors is dealt with from the aspect of the rearing of healthy adults. With the present research such careful adjustment was often neither possible nor necessary, the actual number of adults reared being altogether of secondary importance to the hatching of dormant eggs, and the relative speed of larval growth. Healthy conditions for the larvae occur when they are able to hold bacterial multiplication in check, when they do this very thoroughly their progress is slow and steady, if they hold it slightly in check their development is more rapid, while in cases where the balance of forces is delicate, larval growth is apt to be very rapid, resulting in a life or death race between them and the growth of bacteria. It mattered but very little if this balance, which could not in many cases be readily foreseen, resulted in the destruction of the larvae before the completion of their development owing to the exuberance of bacterial growth. While, although the lagging or eventual starvation of the larvae from lack of sufficient nourishment in some of the more narrowly adjusted experiments is regrettable, it need not lead to any confusion so long as it is borne in mind that the comparison of speed in growth must be made between the larvae in tubes of the same

series and not with those in some other experiments in which the conditions or media differed.

With regard to the hatching of eggs, much preliminary work was done by Bacot in Sierra Leone which is fully detailed in his report (1916), but for the benefit of those who have not had any opportunity of perusing it, the main facts may be briefly recapitulated as follows:

The eggs of *Stegomyia fasciata* are small black spindle-shaped objects of 0.630 to 0.650 mm. in length, by about 0.160 mm. in width if of average size. The shell, which is highly chitinized, is covered with a delicate cellular reticulation and from the central area of a large number, if not of all, the cell spaces rise small rounded bosses, which sections of the egg show to be gelatinous, and not merely the bulgings of the chitin wall of the egg. The eggs are deposited by the females singly, either on the water surface or the wet margins of objects in or surrounding the water surface; no doubt capillary action is responsible for the stranding of many eggs. Incubation is complete after a period of 30 to 50 hours according to the temperature, and Bacot states that it is necessary that the surface of the egg be kept moist while incubation is in progress. McGregor (1916) seems also to have experienced this necessity. After incubation the eggs now containing living larvae may be dried and remain dry for many months, without losing their vitality. Hatching on immersion, after incubation and drying, is generally erratic, a few eggs or a number may hatch within a few minutes; some will take hours, while others will remain dormant under water for days, weeks, and even months, and yet eventually yield healthy larvae.

The *quantity* of water in which the eggs are immersed makes no appreciable difference, nor does the age of the female, or whether the eggs were of a late or early batch. Changing the water in which the eggs were laid had no effect when the fresh water was from the same source and of the same temperature, but a fall in the temperature of the water in which the eggs were lying, exposure to cool air for a few minutes followed by reimmersion in the same water acted as a stimulus to the hatching of a varied percentage of the dormant eggs. The effect of cooling, however, seldom caused more than a small percentage of the dormant eggs to hatch and sometimes had no effect. The immersion of incubated eggs before they had been allowed to dry and others of the same batch after drying, gave divergent results¹.

¹ Although we have not yet followed this point up by planned experiments we have some reasons for supposing that the divergence may be due to bacterial action, during or immediately following incubation.

PRELIMINARY NOTES.

The experiments on which this paper is based were carried out in intervals of time during the course of other work, which in view of the war was of more immediate importance, although the actual delay which resulted from this cause was not serious, because, owing to the slow growth of larvae in some experiments, and the lengthy periods during which eggs remained dormant in others, slow progress was inevitable. From another aspect, however, the length of the gaps, extending in some instances over several months, between an initiatory experiment and its repetition, exerted a considerable influence on the final results, introducing an element of uncertainty as to the nature of the cause to which divergent results were due. For instance, the action of killed cultures and sterile extracts on dormant eggs is not consistent (in an earlier and later series of experiments) and it remains a matter of opinion whether the contradictory nature of the results arises from the treatment of the eggs, the conditions to which the parents were exposed, or the fluctuations in their heredity, which is possibly adjusted to the needs of climatic conditions in the natural habitat of the race of *S. fasciata* experimented with.

The bacterial side of the research was greatly hampered in that one of us had to drop all practical help in research owing to war work at a distance from the Institute, and, although he continued to act in an advisory capacity, it was not possible in his absence to follow various interesting points or to determine the species of the bacteria and yeasts which appeared in the course of the experiments.

It is perhaps necessary to add a word of explanation in regard to the use of the word "sterility." When working with a definite species or group of bacteria for which the suitable conditions of temperature and description of media are charted, the word "sterility" has a definiteness which hardly applies to this work. As here used the word refers to a failure of the organisms to make their presence apparent under circumstances conditioned to the needs of the mosquito larvae. Cultural tests for sterility were carried out with tubes of peptone broth and agar slopes for bacteria; in the case of yeasts with tubes of wort agar. In many cases experimental tubes which repeatedly gave sterile results when the inoculation was performed with a platinum loop were proved to be infected when the test was carried out with a Pasteur pipette which transferred 0.25 to 0.5 c.c., showing how slight the infection really was, either owing to the unsuitability of the temperature or

medium for the organism, or more probably to the clearing action of the larvae. In other cases when infection was suspected owing to the progress of larvae in comparison with their fellows which had hatched from eggs of the same batch in other tubes, it was only proved when cultivations on agar were made with quantities of 0.25 to 0.5 c.c. and the tubes were incubated at 60° to 75° F. It is further significant that larvae from the same batch of eggs might in one tube make no progress at all while in a similar tube perceptible progress would take place, only to cease before the third instar was attained, raising a suspicion of the presence of an infecting organism which either died out or was killed off by the larvae, which subsequently starved. These facts suggested that when eggs hatch the conditions are seldom if ever *really sterile*, but that the organism responsible for the stimulus is unable to colonize the tube owing to small numbers or want of adjustment between itself and the conditions. On the basis of this suggestion the difference between hatching and no growth on the part of the larvae and slight growth preceding death would be explained by the yeast or bacterial infection, probably due to infection from the eggs, dying out immediately in the former instance and more gradually in the latter, possibly owing to the action of the larvae.

Nevertheless, in spite of our frequently correct suspicion, that larval growth indicated infection, there were a few cases in which adult insects were reared under sterile conditions—the final tests being to transfer the dead mosquito from the tube in which it had been reared and died to a tube of peptone broth in which it was allowed to remain for a week or more under a variety of conditions of temperature. In these cases only one or two adults resulted from several larvae, and the possibility remains that the real source of nutriment was obtained from their dead comrades. This last surmise is, however, at issue with the fact that the presence of dead larvae did not in most cases enable the living survivors to complete their development.

We desire to take this opportunity of recording our thanks and indebtedness to our colleagues on the Staff of the Lister Institute for their valuable suggestions and advice, especially to Dr Harriette Chick and Professor Harden, without whose assistance many of the experiments could not have been carried out.

METHODS.

Sterilization. One section of the experiments with eggs and larvae was carried out with unsterilized eggs, and larvae newly hatched from eggs put into clean water which were invariably found to be infected. In the other section attempts were always made to render the eggs sterile before use, and in spite of a number of probably unavoidable failures experience showed that with careful precautions it is quite possible to work under sterile conditions.

The following methods for sterilizing eggs were employed :

(a) Eggs were placed in a minute cotton bag, dipped into 2 % lysol for 5-10 minutes and washed in boiled water. The bottom of the bag was then snipped off and allowed to fall into a tube of broth.

This method gave a few successful results, in many cases, however, sterility broke down.

(b) Eggs were washed in warm (about 90° F.) soft soap and water for 15 minutes, then in 2 % lysol for 5-10 minutes; in boiled water for 5 minutes and then transferred by Pasteur pipette to tubes containing the sterile water or media.

This method gave many successful results, but there were some breakdowns in sterility, chiefly due to moulds and sporing bacteria.

(c) Eggs were washed in tap water by using a fine jet in a deep pan of water; they were then pipetted into weak lysol, about 0.5 %, and vigorously washed round by a jet; after a few minutes they were transferred to 2 % lysol and the stirring by jet repeated. After this they were again placed in 0.5 % lysol and then into boiled water.

Methods (b) and (c) were varied in minor detail from time to time.

(d) A method of sterilization by formalin vapour was tried, but it was too drastically applied in the first instance, the larvae dying on emergence from the egg. Although after suitable development it would possibly have given favourable results, it was not proceeded with, owing to the successful results obtained with (c).

Towards the close of the experiments it was found that the work of sterilization was rendered more certain, and less elaboration of the sterilizing process was necessary if more care was bestowed on obtaining eggs less liable to be covered with a number of species of bacteria, more especially those forming spores. Arrangements were therefore made to rear the parents from which it was proposed to obtain eggs for experiment under conditions which rendered the presence of an infection of sporing bacteria and moulds less likely. A breeding pan

was carefully cleaned and a number of larvae were washed in five changes of tap water, by thorough agitation and violent currents set up by a water-jet; there is little danger of the larvae being washed out of a deep pan, as they invariably keep at the bottom when the water is agitated. They were then left 24 hours, so that they might void the contents of their guts, and then again washed in two or three changes of water. After this preliminary treatment they were put into the clean pan and fed exclusively on broth cultures of *Bacillus coli*. As a result of this procedure the percentage of breakdowns in the sterility of the experimental tubes was much reduced.

One or two precautions with regard to pipetting the eggs are also advisable. Floating eggs should be avoided, and single detached eggs alone should be taken up. When two or more eggs remain attached after immersion in the lysol there is a probability that at the point of attachment a portion of the surfaces will not have been completely sterilized. The reason for avoiding floating eggs is also the danger of incomplete sterilization. In order to get rid of eggs attached to each other, the eggs may be sifted through motor veil gauze or any other very fine gauze.

The whole of the experiments are placed as an appendix, references to them only being given in the text.

SECTION A. EGGS.

Effect of contaminated water. Unsterile conditions.

Experiments I and II, already referred to on page 483, show clearly the powerful action of water charged with organic matter and living organisms on dormant eggs which failed to hatch in clean water.

Effect of alkaline solutions—under unsterile and sterile conditions.

Experiments III and IV. It was suggested by one of our colleagues that the results which followed the addition of water or other media charged with bacteria or yeasts might be due to the ammonia content. Aggramonte showed (1902) that the presence of lye from wood ashes stimulated the early hatching of the eggs. Two trials were carried out, one under unsterile and the other under sterile conditions. From these it seems quite clear that, although a few eggs may respond to the presence of alkaline solutions in either tap or distilled water, the effect is very feeble in comparison with that due to either contaminated water or a broth culture of *B. coli*.

Effect of acidity—sterile conditions.

Experiment IX. Early in the course of the experiments the possibility of the effects resulting from the addition of living bacteria to media such as peptone water or peptone broth being due to acidity was considered. A dilute solution of HCl adjusted to give an acid reaction similar to that in a tube in which the growth of bacteria caused the eggs to hatch produced no response during three days while the eggs were lying dormant in tubes of peptone water and peptone broth, but within 18 hours of the inoculation of these tubes with bacteria all the eggs hatched. A further trial with another tube of peptone water, belonging to the same series, in which the eggs had been dormant for 8 days, also showed a negative result during a period of 48 hours. The acid addition to this tube was increased so as to cause a much more marked reaction than that in the first trial. After inoculation with bacteria one egg hatched within 18 hours and another on the following day, and the remaining two on subsequent days. This halting response was apparently due to the slow and feeble development of the bacteria, owing to the extreme acidity of the tube. The larvae in the tube made relatively very slow progress.

We conclude from the results of the five experiments that the marked response of the eggs was due to the presence of bacteria or their products, apart from either the acidity or alkalinity produced.

Effect of introduction of different living bacteria into tubes of sterile media such as peptone water in which the eggs are lying dormant.

Staphylococcus pyogenes aureus. *Experiment IX* (page 506). A tube of peptone water in which eggs had been lying dormant for 15 days was inoculated from a culture of *S. aureus*. The bacteria failed to grow and the eggs did not hatch. Two days later the inoculation was repeated from the same culture, this time with success and the eggs all hatched within 16 hours.

Bacillus coli communis. *Experiment XI* (page 508). A tube of peptone water in which eggs had remained dormant for 11 days was inoculated from a culture of *B. coli*. All the eggs hatched within 18 hours.

Experiment XX. In a tube of sterile broth, made from dead insects, only one egg out of a number hatched. On the 34th day a quantity equal to about 1 in 7 of filtrate from a broth culture of *B. coli* was added. This had no effect on the dormant eggs, and the tube on

the 39th day was inoculated from a living culture of *B. coli*. The dormant eggs hatched within a few hours. A similar result followed the inoculation of a tube of peptone broth in the same experiment with *B. coli*, the eggs hatching within a few hours of the introduction of the bacteria after they had lain dormant for 39 days.

In Experiment XXIII several instances occur. In a sterile tube of distilled water containing 1 part in 6 of a filtrate from a broth culture of *B. coli*, eggs that had been dormant for 28 days hatched within 12 hours after the tube had been inoculated from a living culture of *B. coli*.

In a sterile tube of distilled water containing 1 portion in 6 of beef broth the eggs hatched on the 50th day within a few hours after the inoculation of the tube with *B. coli*. A similar tube was infected on the 60th day with a small quantity of a broth culture of *B. coli*; the eggs hatched within 10 to 15 minutes. On the 100th day of the experiment eggs that had been lying dormant in a tube of sterile distilled water hatched within 10 minutes of the addition of 3 % of a living culture of *B. coli*.

In a duplicate tube the addition of 3 % of a recently killed culture of *B. coli* caused 1 egg to hatch out of 11 within 15 to 20 minutes, but no more hatched within an hour and a half, when the addition of 3 % of a living broth culture of *B. coli* caused 6 eggs out of the remaining 10 to hatch within 15 minutes. The remaining eggs did not hatch at all and were most probably dead.

Unidentified bacteria.

Cases in which hatching followed the breakdown of sterility were frequent; instances will be found in most of the experiments, in many cases two or more tubes containing different media being affected. Although the species of bacteria were not identified it was clear from their behaviour when sub-cultured that they were of different kinds; some developed spores, while at least three different species were present, which would only grow at a temperature below 80° F.

Apparently any species of living bacterium may act as a stimulus though there is some evidence that certain species act more quickly than others (see Experiment XXIV). It is also significant that hatching only follows inoculation with a small quantity on a loop after several hours, whereas if as large a quantity as 2 % or 3 % of a broth culture is added, hatching follows as a rule within 15 minutes. There is also some evidence which suggests that fresh cultures are more effective than older ones of two or three weeks' age.

Effect of the introduction of living yeast cells.

Experiment XI. To a tube of peptone water in which eggs had been lying dormant for 15 days, about 1 in 5 of a sterile watery extract of brewers' yeast was added. No result followed within one week, when the tube was inoculated from a living yeast culture. All the eggs hatched within a few hours.

Experiment XXII. Some of the eggs placed in a sterile tube of distilled water, with 1 in 6 of beef broth added, remained dormant for 28 days. A small mass of cells from a culture of *Saccharomyces cerevisiae* on wort agar was added, when the dormant eggs hatched within a few minutes.

In the same experiment 7 tubes of distilled water, in which the eggs had lain dormant for 28 days, were subjected to the same treatment, most of the eggs hatched within 5 to 10 minutes.

Experiment XXIII. Eggs that had been lying dormant for 28 days in a tube of sterile distilled water, containing 1 in 6 of the filtrate from a broth culture of *B. coli*, hatched within 10 minutes of the introduction of a small mass of living cells of brewers' yeast.

In the same experiment (XXIII) on the 100th day 3 % of sterile autolyzed yeast was added to a tube of sterile distilled water in which there were dormant eggs. One egg out of 9 hatched within 15 to 20 minutes; after an hour and a half, during which period no further eggs hatched, a small mass of living yeast cells from a wort agar culture was added, and the remaining 8 eggs hatched within 15 minutes.

These examples afford clear evidence of the stimulus to hatching exerted by living yeast cells. In Experiment XI the yeast used was one that had been isolated from a human throat, but in the other tests *S. cerevisiae* was used.

Effect of the presence of living moulds.

No special tests were carried out with moulds, but during the course of the experiments it was remarked that when a mould developed in an otherwise sterile tube many if not all the dormant eggs hatched. It is apparently possible for moulds to exert this influence when very small and quite inconspicuous. In a number of cases in which eggs had lain dormant for several days their hatching was quite inexplicable until a tiny white speck, which proved subsequently to be a growing mould, was noticed. A microscopic examination in one such instance showed the hyphae ramifying over the surface of the egg shells. Many ex-

amples of hatching in apparent response to moulds occurred in Experiment XXII.

The most plausible suggestion as to the nature of the stimulus which induces the eggs to hatch would seem to be that of a *scent* which penetrates to the larvae lying dormant within the egg shells causing them to make vigorous movements resulting in the uncapping of the egg. There is, however, a difficulty in the way of the acceptance of this theory, owing to the partial or entire failure in some cases of killed cultures or filtrates of *B. coli* and sterile yeast extracts to bring about the hatching of the eggs. This subject is dealt with in greater detail in the following section.

The effect of killed bacterial cultures; sterile filtrates of B. coli and extracts of brewers' yeast.

Until nearly the close of the experimental work no results had been obtained from the addition of killed cultures and sterile filtrates of *B. coli* (see Experiments XIV, XXI and XII). On one occasion (Experiment IX) the addition of about 17 % to 20 % of a sterile autolyzed extract of brewers' yeast caused eggs which had been lying dormant in a tube of peptone water for a month to hatch within one hour, but the larvae died within a few minutes. In Experiment XI about 8 % of a sterile watery extract of brewers' yeast was added on the 15th day to a tube of peptone water containing dormant eggs, but no action having resulted within a week, the tube was inoculated from the culture of a species of yeast isolated from a human throat. The eggs hatched during the following night.

When sterilized eggs were pipetted into tubes of distilled water containing 2 % or 3 % of an autolyzed extract of brewers' yeast (Experiments XVI and XVII) hatching was not general, nor did hatching occur any more freely than in different media in the other tubes. In Experiment XVII larvae continued to hatch out from dormant eggs over long periods. In one case eggs that had remained dormant for over 90 days hatched without any interference with the tube. While in another instance eggs that had been resting for 126 days hatched on the addition of boiled distilled water to three tubes which were almost empty owing to evaporation.

Experiment XXI. None of the eggs in two tubes of an autolyzed extract of brewers' yeast solution (3 % in distilled water) hatched within 96 hours, but one larva emerged in each tube within 120 hours. In one tube no more eggs hatched, but in the other two or three larvae

emerged and died before the 12th day; both tubes were then inoculated from a living culture of *S. cerevisiae* and all the remaining eggs hatched during the night.

In spite of the foregoing negative evidence we thought it desirable to test the matter further, because it seemed possible that the failure might be due to either too high a temperature in the sterilization process or to the yeast extract not being sufficiently fresh—some days having been allowed to elapse between preparation and use.

With this object Experiment XXIV was planned. A number of tubes of distilled water containing eggs were used on the fifth day after the addition of the eggs. Cultures of *B. coli*, *S. aureus* and autolyzed extract of brewers' yeast which had been autoclaved (120° C.) were used, also cultures of *B. coli* and *S. aureus* which had been killed by steam heating at 100° C., a culture of *B. coli* killed by chloroform and a sterile filtrate of *B. coli*. The addition to the tubes was 6 %, except that in one of the tubes only 2½ % of the yeast extract was added, but this alteration made no difference to the result. It will be seen that in contradistinction to the earlier results the sterilized cultures and yeast extract were effective, but not the filtrate. This last was several weeks old at the time of use, but fresh filtrate had been used in the earlier trials without effect. It is interesting to note that the eggs were slower in responding to the *S. aureus* than to *B. coli* in all four tubes.

The results were so generally at variance with those in the earlier trials that it seemed probable that the difference must lie in the eggs. With a view to testing this hypothesis advantage was taken of several tubes of distilled water in Experiment XXIII in which eggs had been lying dormant for 100 days. These were tested by adding a quantity equal to about 3 % of a living culture of *B. coli*, autolyzed yeast sterilized by autoclave and *B. coli* sterilized by autoclave, the last two being from the same tubes as the additions to the tubes in Experiment XXIV. It will be seen that the results again differ, being more in line with the early trials, but not quite in conformity. There seems no doubt but that the susceptibility of the eggs varies greatly while it is also consistent with the results to suppose that the cultures but not the filtrate used—possibly because of their freshness—were more effective.

A further trial, Experiment XXV, was carried out to test the watery extract of yeast as against an autolyzed one, and both as against living cells from a culture of *Saccharomyces cerevisiae* on wort agar.

As a whole this test shows clearly that while, for these particular eggs, the autolyzed extract and the living cells were almost equally

effective, the watery extract was far less so, and in one tube had no effect at all within three-quarters of an hour. The difference in the results in the several tubes must be taken as measure of the variable susceptibility of the eggs. It will be noted that in one of the tubes to which the *living yeast cells* were added, there was only a partial response within the first three-quarters of an hour. It is regrettable that no tubes containing eggs which had long lain dormant were available to control results, as was done with the previous trial (Experiment XXIV).

In discussing these results it is necessary to note that hatching may follow if during the sterilizing process the eggs are left too long in lysol. Sometimes these eggs do not uncap until after their transference to water, but in both cases the larvae fail to come right out of the shell. The same phenomena followed when eggs were exposed to formalin vapour for several hours. After transference to water large numbers uncapped, but the larvae only partially came out of the shells. In these instances no movement on the part of the larvae was ever observed. It is possible that they were killed within the egg, but cut or partially cut the cap in their dying struggles. There is a difficulty in the way of the acceptance of this explanation, however, because it is not easy to understand why, in the case of those placed in lysol, the uncapping should sometimes be delayed until after they were transferred to water.

The differential hatching of the eggs may, as pointed out in the introductory notes, arise as an hereditary trait owing to an adaptation to meet the needs of seasonal changes. On the other hand there is evidence that treatment during incubation or prior to full immersion may be the cause of more ready response to immersion. Bacot (1916) found when in Freetown that of a given batch of eggs, which had incubated on a wet surface, 98 % of those which were immersed without allowing them to dry hatched within 30 minutes; of a portion of the same batch placed in water after 24 hours' drying the hatching of 84 % was distributed over a period of 65 days; while the balance of the batch, which was kept dry for 7 days before immersion, again showed a divergent result, 43 % hatching within 24 hours, another 11 % following within 51 days and of the balance 8 % containing living larvae when dissected after 81 days.

In Experiment XVI the eggs were not allowed to dry before sterilization, as it was desired to test the theory that a check in the influence of bacterial action on the eggs might be the cause of delay in their hatching when subsequently immersed. Although the larvae

did as a matter of fact emerge rather freely from these eggs under sterile conditions, the evidence from Experiment XXIII, in which also they were not allowed to dry before sterilization, is entirely negative, the number hatching under sterile conditions being small; while in Experiment XXV in which dried eggs were used the percentage hatching in some of the tubes was considerable. It is probable that the problem is a complex one, two or more factors being concerned.

The results of the experiments on eggs is so far clear and decisive in regard to the fact that the presence of bacteria, yeasts and, less definitely, moulds, does exert a stimulus causing eggs to hatch that would, apart from their presence, have remained dormant for a longer period. It also seems definite that this stimulus is less powerful, or may be altogether ineffective, if killed cultures or sterile filtration and extracts of bacteria or yeasts are used. The difference is apparently one of *quantity* not *quality*, its extent being chiefly dependent upon some variation in the susceptibility of the eggs.

SECTION B. LARVAE.

Unsterile conditions.

The fact that the larvae greedily consume clumps of bacteria or yeasts (*S. cerevisiae*) removed from agar cultures has already been mentioned in the introductory remarks. This rough test is open to the objection that what the larvae struggled for was not the organism but the organic matter on which they were living, or by-products produced in their development.

In Experiments V and VI, *B. coli* washed by centrifuging were given as food to a batch of larvae, while to an equal number in a similar quantity of water no food was supplied; the unfed batches constituting controls. The results show conclusively that the larvae cannot only live, but thrive on the bodies of bacteria.

Sterile conditions.

These early trials, followed by a series of experiments (VII, VIII, IX and XI), afford examples, in which the eggs were sterilized and transferred by Pasteur pipette into tubes containing various sterile media; the object being to ascertain if it was possible for the larvae to develop under sterile conditions. In the main the results are clear and decisive; while sterility is maintained, larval development is either inhibited or proceeds excessively slowly in conjunction with a high, or

more frequently a total mortality. The infection of a tube with bacteria is generally followed by larval progress, usually rapid. The only exceptions are cases in which the larvae had existed under sterile conditions for long periods and seemed incapable of adjusting themselves to the change. Exceptions to the general rules of rapid larval development in infected tubes are most noticeable in Experiments XII and XIII when the bacteria which caused the breakdown in sterility proved to be feebly growing species, which altogether failed to keep pace with the larval requirements. In Experiments XI, XVI and XVII adults were reared under sterile conditions, the greatest success (in Experiments XI and XVI) being attained in a medium of pure beef broth (without the addition of salt or peptone) which for some unexplained reason seemed favourable for slow but sustained growth. Two adults were reared in separate tubes of a 3 % solution of an autolyzed extract of brewers' yeast in distilled water (Experiment XVII). One of these was reared under sterile conditions and the other ought, properly speaking, to be credited to these conditions as, although a mould was present in the tube when the adult emerged, all the evidence is against any suggestion that the larvae obtained any nutriment from this source—the presence of moulds being seemingly inimical or neutral to larval development; in this particular instance all the remaining larvae in the tube died.

While in these, and in fact in all, cases of larval progress under what are apparently sterile conditions, the possibility must not be excluded of a slight infection occurring during a portion of the larval existence and then dying out, the only evidence in support of such a supposition is the fact that larval progress varied in what were apparently sterile tubes independently of the media. This has happened in one or more out of a batch of tubes of the same medium, the eggs being part of a number all sterilized together. Taking into consideration the variability exhibited by the eggs at a stage when they contain fully developed larvae, it will require a considerable body of positive evidence based on carefully planned experiments before such a possibility can be accepted as a probability.

In Experiment XI a test was carried out to see if a change from one sterile medium to another had any effect in stimulating larval growth. Of two larvae withdrawn on the 15th day from a sterile tube of peptone broth, one was transferred to a tube of broth made from dead insects and the other to a tube of water in which horse dung had been steeped. After a pause of some 48 hours some evidence of quickened growth was

observed, but the progress was not continuous and both larvae died some 50 days later without having completed their larval development.

Experiments XII and XIII were designed with a view to trying sterile media composed of substances more likely to be met with by mosquito larvae under natural conditions than those normally used for bacteriological purposes. Tubes containing water in which horse dung had been steeped 48 hours previously and a broth made from dead insects—chiefly flies—were used. The series afforded but little evidence bearing on the original purpose of the tests owing to the infection of the tubes by obscure species of bacteria whose presence was difficult to detect, and such evidence as was forthcoming was negative. The tests convinced us, however, of the unsuitability of these media which were likely, owing to the sources from which the nutriment contained in them was drawn, to harbour sporing bacteria.

Particulate matter. One of our colleagues suggested the possibility of the necessity of particulate matter in the medium as it seemed possible that larvae swimming in a true fluid might be unable to pass it through the alimentary system in the absence of solid particles in the intestine. We accordingly added powdered animal charcoal to a number of tubes of a solution of autolyzed yeast in distilled water (*Experiments XVI and XVII*) with apparent success at the outset, as the larvae not only ingested it—the dark gut content being clearly visible—but made quicker progress than others in the same media without the charcoal. The advantage did not, however, continue and, although one reached the pupal stage, no adults were reared; on the other hand, as already recorded, one adult was reared from a sterile tube of the yeast extract solution, without the charcoal. It is also to be noted that the insect broth which contained much solid matter also failed, under sterile conditions, to confer any advantage after the first few days.

A few trials were carried out to test if milk acidified to such a degree that the colloidal particles of the casein were increased in size but not precipitated and white of egg precipitated as very minute particles, would afford the necessary pabulum for the larvae. After sterilization small quantities of these preparations were added to media in which eggs had already hatched, but in which the larvae were making no progress, or the larvae were transferred by Pasteur pipette to tubes containing milk or white of egg in this form. Examples will be found in *Experiments XIII, XVIII, XIX and XX*. In no case did the addition or presence of the substances enable the larvae to develop

while sterility was maintained; although in some instances slight progress followed the addition (Experiment XIX, page 518), it soon ceased, the larvae eventually dying without attaining their full growth.

Killed cultures of B. coli.

In Experiment X a preliminary trial was made to ascertain if the larvae could be reared under sterile conditions upon a culture of *B. coli* killed by heat. No success followed, but young larvae hatched from unsterilized eggs made rapid progress in a control tube, this tube being of course infected by the larvae. Experiment XIV was a repetition on a larger scale; several tubes were prepared, but the eggs placed in these failed to hatch. A single newly hatched larva (the only one which could be spared at the moment) was transferred from another experiment to one of the tubes. It failed to make any progress during a month and died within a few hours of the inoculation of the tube at the end of this period.

Sterile filtrate of B. coli.

In Experiment XXIV a culture of *B. coli* killed by chloroform was added to tubes of sterile distilled water containing eggs; a number of these hatched and tube No. 12 remained sterile, although its fellow No. 11 became infected on the 11th day. None of the larvae in No. 12 passed the first moult, while some of those in the infected tube had made considerable progress and were in the third instar. The larvae in tube No. 12 all died in the second skin, whereas in the infected tube the larvae were well grown before the food supply failed, two at least having attained their fourth (last larval) instar.

Thinking that possibly the action of the heat used in killing the cultures of *B. coli* might have the effect of destroying its nutrition value for the mosquito larvae, a number of trials were carried out with the sterile filtrate from broth cultures of *B. coli*. No success attended any of them, either in neat or diluted condition, nor did the addition of the filtrate to other sterile media in which larvae were living produce any effect, apart from the few cases in which sterility broke down at the period when the filtrate was added. (See Experiments XIX, XX and XXIII.)

Living yeast and yeast extracts.

See Experiments XVI, XVII and XXI; note also the conclusion of XXII, page 521, of XXIII and of XXIV and the conclusion of XXV. There is a break of similar character between the nutritive value of

living *S. cerevisiae* and sterile extracts of brewers' yeast to that which separates living from dead cultures, or sterile filtrates of bacteria. It is not quite so definite, however, as the larvae do make progress in solutions of autolyzed yeast extract, whereas throughout the whole course of the experiments no larval progress has occurred in sterilized cultures of bacteria or sterile filtrates. The reason for this difference is not obvious and it seems possible that by some system of concentrating and extracting bacterial cultures analogous to the autolyzing process with yeasts, the difference might be eliminated.

The effect of the presence of moulds on larval growth.

Although no experiments were planned with a view to testing the influence of moulds, their occurrence in both sterile and bacterially infected tubes was so frequent in some of the experiments that a considerable amount of general observation was possible. The impression gained during the progress of the research was that moulds were inimical to larval development, but a careful consideration and comparison of the notes show that the fatality which so frequently followed their appearance in the experimental tubes cannot be clearly demonstrated as due to their presence. In a few cases of rapidly spreading surface moulds it is extremely probable that they were an accessory, if not the actual cause of death, and in Experiment XXII an instance of mortality apparently due to moulds occurred in tubes Nos. 4 and 5 of the distilled water series. What does, however, appear quite definite is that they are of no service whatever to the larvae as a source of food supply. In fact it seems reasonable to suppose that they may compete with the larvae for whatever supply of nutriment is available. Although the evidence on this point is not altogether consistent, in Experiment XXII it appears quite negative, there were several instances in which fully grown larvae died in tubes where moulds had developed. In a few cases one or two out of several of the larvae succeeded in completing their development, the others dying rather inexplicably if the moulds were not implicated. Two or three instances occurred of moulds growing from the mouths of dead larvae, as though swallowed spores had developed in the alimentary canal; while there is of course no evidence that the development of the mould preceded death, the spores must presumably have been ingested by the living larvae. Notes referring to the presence of moulds will be found in Experiments XXI, XXII and towards the close of XVII and XXIII.

Our conclusion respecting the evidence as a whole may be stated as follows: that the larvae of *Stegomyia fasciata* greedily consume both bacteria and yeasts on which they can thrive in the absence of any other food, whereas in very many instances they fail entirely to develop on a variety of nutritive fluids and particles, including dead bacteria, under sterile conditions. In the far less numerous cases where progress was made under apparently sterile conditions, growth was always relatively very slow to that under otherwise equivalent but unsterile conditions, and the mortality was very high. The rearing of adults under sterile conditions being so exceptional we feel justified in stating that the presence of bacteria or yeasts is a practical necessity for the maintaining of the species. If one considers the facts shown by Bacot (1916) regarding the scarcity of bacteria in the alimentary canal of larvae taken from water swarming with bacteria in relation to the exclusively browsing habit of the young larvae and its partial retention by the older ones when feeding, together with the experimental results above recorded, there seems good reason for supposing that bacteria and yeasts afford the chief food supply of the larvae. The ingestion of larger particles and the structure of the jaws are not incompatible with this view, because such particles are likely to be covered with a bacterial growth while the jaws are of use in gnawing away portions of decaying organic matter. They were evidently used to disrupt clots of both bacteria and yeasts in the preliminary experiments mentioned in the introduction.

It seems probable that this knowledge may be of assistance in the destruction of this species of mosquito, as it should enable the methods now so largely available for the purification of water from bacteria to be utilized. On a minor issue it may be of service in enabling Sanitary Officers to escape the annoyance caused by the failure to breed more than one or two isolated species from jars containing mosquito larvae captured by their inspectors. This failure no doubt arises owing to starvation, because the bacteria are killed by the action of light, it being customary to stand jars with wrigglers in the full light of a window. There is the further possibility that the eggs after a careful and probably lengthy research might be found to be of service as a rough and ready means of testing the relative purity of water in regard to living organisms.

EXPERIMENTS WITH UNSTERILIZED EGGS.

Effect of foul water charged with bacteria.

EXPERIMENT I.

100 c.c. of tap water was put into each of two glass pans. The water temperature during the course of this experiment was about 65° to 67° F.

A batch of about 350 eggs laid on filter paper three weeks previously and allowed to dry after incubation, but stored in a moderately humid atmosphere, was divided into two lots, approximately half being immersed in each pan.

To the water in one pan (*A*) was added 3 c.c. of sewage water (a fragment of human faeces was placed in water and allowed to incubate for 48 hours at 80° F.); to the water in the other pan (*B*) nothing was added.

After 1 hour in <i>A</i>	4 eggs had hatched.				
" " <i>B</i>	no eggs had hatched.				
After 4 hours in <i>A</i>	10 additional eggs had hatched.				
" " <i>B</i>	no eggs had hatched.				
After 20 hours in <i>A</i>	113 additional eggs had hatched.				
" " <i>B</i>	6 eggs had hatched.				
After 70 hours in <i>A</i>	9 additional eggs had hatched.	Total	136.		
" " <i>B</i>	36 " " "	Total	42.		

EXPERIMENT II.

The experiment was repeated, using water in which horse dung had been steeped for the stimulating fluid. The quantities and other arrangements were similar to those in Experiment I, 3 c.c. of the contaminated water was added to pan *A* at the start, but none to pan *B*.

After 18 hours in <i>A</i>	148 eggs had hatched.				
" " <i>B</i>	11 " "				

3 c.c. of the contaminated water was then added to pan *B*. At the expiration of another 18 hours 159 additional eggs had hatched in pan *B*. The size of these larvae suggested that they must have hatched within an hour or two of the addition of the manure water.

EXPERIMENT III.

*Contrasted effect of alkali and water charged with bacteria.**Temperature 75° F.*

Into each of 4 pans containing 100 c.c. of tap water was placed .007 gr. of incubated eggs of *S. fasciata* (the number would be about 200 or 300 eggs). The pans were kept all night at 75° F. Pan (1) formed a control. To pan (2) was added a solution of washing soda, producing an equivalent of 1 in 1000 in the pan; to pan (3) sufficient ammonia was added to produce 1 in 1000. To pan (4) about 5 c.c. of peptone broth from an experimental tube in which a bacterial infection caused the eggs to hatch.

After 6 hours (No. 2) Soda, showed a few larvae out.

„ (No. 3) Ammonia, showed a few larvae out

„ (No. 4) Contaminated, many.

„ (No. 1) Control, only one or two.

To the control pan about 2 c.c. of an infusion from horse dung was added, and the pans were left all night.

18 hours later (No. 2) Soda, showed a small number out.

„ (No. 3) Ammonia, about the same number out.

„ (No. 4) Contaminated, swarms.

„ (No. 1) Control, after the addition of the infusion of horse manure many more had hatched than in either the soda or the ammonia containers, but not so many as in the contaminated pan.

10 c.c. of water from pan (No. 4) was added to pans Nos. 2 and 3. An examination 6 hours later showed that the numbers of larvae had increased by one-third to a half, the freshly emerged larvae being easily distinguished from those which had hatched some hours previously, by their pointed conical heads.

EXPERIMENT IV. *With sterilized eggs.*

Contrasted effect of alkali, filtrate of B. coli and living B. coli.

Tubes taken from Experiment XXI (see page 519).

A batch of eggs was sterilized by Method *b* and from 12 to 20 were pipetted into a number of tubes containing 10 c.c. of sterile distilled water and one (No. 5) containing sterile filtrate of *B. coli*. After 20 hours at 75° F. the tubes were examined and four containing distilled water were selected in which only one or two eggs had hatched, the idea being to use resistant eggs which did not respond without stimuli.

„ In tube No. 1, 2 eggs had hatched (1 larva was dead).

„ No. 2, 2 „ „

„ No. 3, 2 „ „

„ No. 4, 1 „ „

„ No. 5, 0 „ „

To No. 1 was added 1 c.c. of water in which horse dung had

been steeped for 48 hours at 37° C. ... = 10 %.

„ No. 2 was added 1 c.c. of a sterile filtrate of a *B. coli* culture = 10 %.

„ No. 3 „ 1 c.c. of a 1 % solution of washing soda = 1-1000.

„ No. 4 „ 1 c.c. of a 1 % solution of .880 ammonia = 1-1000.

No. 5, a tube of filtrates of *B. coli* culture, was inoculated from a living culture of *B. coli*.

Result after 1 hour:

						Increase in hatching
No. 1,	more than 12 larvae out	...	...	...	...	10 or over
No. 2,	4 larvae out	...	...	...	...	2
No. 3,	3 „	...	...	...	...	1
No. 4,	1 „	...	...	...	...	0
No. 5,	0 „	...	...	...	...	0

After 24 hours:

						Increase in hatching
No. 1, more eggs had hatched—probably all.						
¹ No. 2, 8 larvae out	...	...	...	...	...	6
No. 3, 3	„	...	...	...	...	1
No. 4, 2	„	...	...	...	...	1
No. 5, 12	„	...	...	...	...	12

After 68 hours:

- No. 1, all the eggs hatched, larvae have grown rapidly.
 No. 2, 9 larvae out—slight progress.
 No. 3, 4 „ no progress.
 No. 4, 2 „ both dead.
 No. 5, all (12) larvae out, have grown rapidly.

The strength of the soda and ammonia solution in tubes Nos. 3 and 4 was doubled to 1 in 500.

After 24 hours no further eggs had hatched.

To each tube, Nos. 2, 3 and 4, .5 c.c. of a culture of *B. coli* was added.

One hour later: all the remaining eggs in tubes 2, 3 and 4 had hatched—

- No. 2, 12 or 13 living larvae.
 No. 3, 12 or 14 living larvae.
 No. 4, 12 living larvae.

The larvae in No. 4 (ammonia 1 in 500) all died within 24 hours.

Two days later the larvae in tubes Nos. 2 and 3 had made very rapid progress and had caught up to those in tube No. 5 in the matter of growth. They actually passed them during the next day or so and then slowed down. The larvae in all these tubes came to a standstill within 10 days, having apparently exhausted their food supply.

EXPERIMENT V (*unsterilized conditions*).

Ability of the larvae to develop on a diet of bacteria.

Temperature 75° F.

Bacillus coli was washed by centrifuging 4 times in succession and a small quantity of the precipitate placed in 30 c.c. of tap water with 5 newly-hatched larvae.

Control. 5 newly-hatched larvae of the same batch were placed in 30 c.c. of tap water, minus the *B. coli*.

The control larvae marked time with little or no progress; 1 larva died on the 6th day and 1 was feeble and died on the 8th day. By the 11th day 2 of the larvae passed the first moult, while a third larva died on the 12th day. After 17 days 1 larva attained to its third instar; both the larvae were living after the 30th day, but were dead by the 37th day. The larvae given *B. coli* grew rapidly; by the 3rd day 2 of the larvae had passed the second moult and the others were nearly full grown in their second instar.

¹ It is probable that tube No. 2 was already infected as numerous later tests of *B. coli* filtrate added to tubes in which dormant eggs were lying, showed that the filtrate did not stimulate hatching.

The supply of washed *B. coli* in the pan was renewed. By the 6th day all the larvae were in the fourth instar. One pupated on the 8th day, and the remainder had pupated by the 10th day. Two ♀♀ specimens emerged on the 12th day and two more ♀♀ and a ♂ by the 13th.

The pan was then restocked with 5 newly-hatched larvae, but no fresh food given. All the larvae were in their second instar by the 3rd day; they reached the third instar by the 7th day, having exhausted the food supply, and were still in the same stage 10 days later.

EXPERIMENT VI (*unsterilized conditions*).

Ability of larvae to develop on a diet of bacteria.

Temperature 75° F.

Into one of two beakers, each containing 200 c.c. of tap water, was placed sufficient of a washed culture of *B. coli* to cloud the water.

20 newly-hatched larvae were placed in each beaker, and card covers placed on top to exclude dust.

Control. No appreciable progress, beyond the expansion of the head, took place within a week, then 8 died within 24 hours. Seven days later only 6 of the 20 larvae were living, 3 of which had passed the first moult. After another week (21 days from start) these 6 were still living, and making slow progress.

After 75 days there were two survivors, which by this time were slender larvae in the third instar. A little water mixed with faeces and therefore containing bacteria was added to the pan. Rapid growth was noticeable by next day; 1 larva had pupated within 5 days, and by the 9th day—84 days after hatching—2 adults were bred.

The larvae given *B. coli* made rapid progress; all were in the third instar by the 4th day; 16 had pupated by the 9th day; 4 ♂♂ and 1 ♀ emerged on the 11th day and the remainder followed within a few days; the last, which had lagged in the larval stage, taking 16 days.

Experiments under sterile conditions in tubes containing nutrient media.

EXPERIMENT VII.

Eggs sterilized by Method *a* were pipetted into a tube of peptone broth, after a night at 75° F., the tube was allowed to cool to 60° F., and some of the eggs hatched. The larvae showed no sign of growth after 48 hours at 75° F. The tube was then infected with *B. coli*; within 18 hours at 75° F. the broth was cloudy and the increase in size of the larvae was very obvious.

A curious feature of the experiment was that small bubbles of gas which were liberated by the fermentative action of the bacteria, formed on empty eggs, fragments of egg-shell etc. lying at the bottom of the tube, floating them up to the surface, when, the bubble becoming detached, they sank to the bottom and the process was repeated. Similar bubbles formed at the apex of the syphon tubes of the larvae, causing them to float upwards in spite of their struggles to swim downwards. So regular and constant was this process that the larvae got but little time for undisturbed feeding, and their growth was slow in comparison with their rapid start.

After 4 days the formation of gas ceased and the larvae again commenced to grow rapidly, and within 3 days some had attained their third skin. The tube, however, became very foul and all the larvae were killed.

EXPERIMENT VIII.

Eggs sterilized by Method *a* were pipetted into a tube of peptone water and a tube of beef broth without either salt or peptone.

No eggs hatched in either tube in response to the conditions and lapse of time, which were successful in the first trial.

Broth. After 48 hours sterility broke down in the broth tube; many of the eggs hatched and the larvae made rapid progress, 4 days later some of the larvae passed their third moult, but others were much behind on the 24th day of the experiment; 22 days after hatching the first adult was reared and others followed, but there was a heavy death-rate, presumably owing to there being too gross a growth of bacteria in the tube.

Peptone water. This tube remained sterile and, in spite of warming and cooling, no eggs hatched until the 3rd day, when one larva emerged; others followed and 5 or 6 were seen by the 5th day. Growth was not quite inhibited, but hardly any progress had been made by the 4th day after hatching, the larvae being still in the first instar.

On the 8th day of the experiment, 4 days after the hatching of the eggs, sterility broke down, the tube becoming cloudy. The larvae emerged from the remaining unhatched eggs and rapid progress commenced.

The larvae made rapid growth at first, and, owing to their number or the less vigorous growth of the bacteria, the conditions in the tube remained healthy, the larvae keeping the clouding of the peptone water in check and finally clearing it; they then began to find a shortage of food and ceased to grow, finally dying on the 24th day of the experiment—the last to die having reached the third instar.

EXPERIMENT IX.

(Includes notes respecting the addition of acid on dormant eggs.)

Eggs were sterilized by Method *b* and pipetted into four tubes of peptone water and four tubes of broth, only 5 or 6 eggs being placed in each tube.

After 48 hours, 4 eggs had hatched in one of the tubes of broth (No. 1) and in this tube only had sterility broken down; a 5th larva emerged in this tube next day. A plate culture was made from this tube, and a sporing bacillus isolated. On the evening of the 3rd day from the start one of the broth (No. 2) and one of the peptone tubes (No. 3) in which no eggs had hatched, were inoculated from the tube which had become infected. By next morning 4 larvae had hatched in the broth and 1 in the peptone tube. None hatched within the next 2 or 3 days in the remaining sterile tubes. The following test was then made: to two of the sterile tubes—broth No. 4 and peptone No. 5—was added sufficient of a dilute solution of HCl to produce a similar acid reaction to the broth tube in which sterility broke down. No response took place within 20 hours, but the remaining eggs in the inoculated peptone water hatched within this period.

By the 7th day of the experiment the larvae in the broth tube in which sterility broke down were all in the fourth instar, while those in the inoculated broth tube

were in the second and those in the inoculated peptone water in the third instar. No eggs had hatched in the tubes to which acid had been added 3 days previously and the tubes were now inoculated from No. 1; within 18 hours all the eggs in them hatched.

Another trial was made, with dilute acid of higher concentration, in one of the peptone tubes (No. 6) in which the eggs had now lain dormant for 8 days; the acid reaction after the addition being much stronger than in the original broth tube (No. 1).

After 48 hours, during which period no larvae hatched, the third tube to which acid was added (No. 6) was inoculated from tube No. 1; by next morning (18 hours later) one egg had hatched, another larva emerged on the 2nd day, and 2 others on following days. The bacterial growth in this tube was feeble, presumably owing to its relatively strong acidity, while the larvae in it made very little progress.

On the 14th day of the experiment two eggs were taken by means of a Pasteur pipette from the remaining sterile broth tube (No. 7) and transferred to a tube of peptone broth; they lay unhatched in the tube of peptone broth for 8 days, when the tube was inoculated with *Staphylococcus aureus*; within 16 hours larvae had emerged from both the eggs. They died, however, within 3 days, apparently owing to the vigour of the bacterial growth.

On the 15th day of the experiment, the broth tube from which these eggs had been removed (No. 7) was inoculated with *S. aureus*. The culture did not grow and the eggs failed to hatch. Two days later the inoculation was repeated from the same culture; this time with success, and the eggs which had been lying in the broth for 17 days hatched within 16 hours.

On the 32nd day of the experiment 1 c.c. of sterile autolyzed extract of brewers' yeast (= 17 % to 20 %) was added to the remaining tube of peptone water (No. 8) in which the eggs had been lying dormant for a month. The eggs hatched within an hour, but the larvae died within a few minutes of their emergence.

History of the larvae in the above experiment.

Tube No. 1; eggs hatched in response to casual infection.

The larvae were in the fourth instar by the 7th day; 4 days later the first pupa was noted and 3 days later the first pair of adults emerged; 2 ♂♂ and 3 ♀♀ followed within the next day or two—15 days from hatching.

Tube No. 2 broth and No. 3 peptone eggs hatched on the 3rd day of experiment. The bacterial growth was more vigorous in the peptone water than in the pure beef broth. Four days after the hatching of the eggs the larvae in tube No. 2 were in their second instars, while those in tube No. 3 were already in the third skin. This rapid progress on the part of the larvae in tube No. 3 (peptone water) did not continue long; the dense growth of bacteria rendered the conditions unhealthy and 4 days later the larvae in tube No. 2 were ahead of those in tube No. 3, while the latter were beginning to die off.

On the 11th day from the hatching of the eggs all the larvae in tube No. 3 were dead, while two of those in tube No. 2 had pupated. The remaining larvae were full fed and the broth was bright and transparent, the number of bacteria having been reduced to a minimum either as a result of the larvae devouring them or organic matter in the broth necessary for bacterial development.

Tubes Nos. 4 and 5. Four days after hatching, the larvae had made good progress, those in the broth tube being well ahead, while by the following day the growth of bacteria in the peptone water had become exceedingly dense and one or two of the larvae were dead. By the 7th day after hatching 4 larvae in the broth tube had pupated, while the surviving larvae in the peptone water were still in their third instar. Five days later (12 days from hatching) all the larvae in the peptone water were dead, and a pair of adults had emerged from the pupae in the broth tube; the remaining pupae and larvae died.

Tube No. 6. These larvae made very slow progress; the growth of bacteria in the tube being feeble. By the 6th day after hatching they had only attained to their second instar. Five days later the larvae were still all living and had made steady progress; one pupated on the 13th day from hatching and 2 ♂ emerged on the 17th day; 2 ♀ following on the 18th and 20th days.

Tube No. 7 (broth). Four days after hatching only 2 of the 4 larvae were living; these had made rapid progress and were in their fourth instar; 2 ♂ specimens were bred on the 11th day from hatching.

EXPERIMENT X.

Washed B. coli killed by heat as food for the larvae.

A small quantity of washed *B. coli* was put into two tubes of distilled water which were sterilized by heat.

Into one tube a larva was transferred from a tube of peptone water that had remained sterile for several days; into the other several larvae that had just emerged from unsterilized eggs placed in tap water. The cloudiness which followed in this tube indicated that the larvae had infected it. The growth of these larvae was rapid; on the 5th day they were well grown in the third instar—the sterile larvae having made barely appreciable progress in the first instar.

The progress of the infected larvae slowed down gradually as the water cleared, but they continued healthy.

In the sterile tube the larvae progressed very slowly and died after moulting on the 9th day.

EXPERIMENT XI.

(Includes notes concerning the effect of sterile yeast extracts on dormant eggs and a change of diet, under sterile conditions, on the larvae.)

Five to 7 eggs sterilized by Method *b* were transferred by pipette into the following tubes: 4 of peptone broth; 3 of peptone water; 3 of beef broth (no salt or peptone); 1 in boiled tap water and 1 in boiled distilled water.

After 24 hours eggs left in the lysol used in the sterilizing process had hatched; the larvae were dead and many had failed to get free of the eggshell. Only one egg hatched out of a number that were transferred from the lysol to tap water. Several eggs out of a large number left in the flask of boiled water hatched. Some eggs also hatched in the tubes of peptone broth, broth tubes, and in one of the peptone water tubes, but none in the boiled tap water or boiled distilled water.

After 48 hours one larva hatched out in the boiled distilled water; none in the boiled tap water.

During the same period in peptone water two larvae came out of 6 eggs in one tube only.

<i>Beef broth.</i>	Tube (No. 1), 1 out of 7 eggs
	„ (No. 2), 3 „ 7 „
	„ (No. 3), 4 „ 7 „
<i>Peptone broth.</i>	Tube (No. 1), 5 „ 6 eggs (1 larva dead)
	„ (No. 2), 4 „ 6 „
	„ (No. 3), 2 „ 5 „
	„ (No. 4), 2 „ 6 „

The growth of the larvae was negligible.

Three days later (5 days from start):

Boiled tap water. None had hatched.

Boiled distilled water. No progress by the larva.

Peptone water. No further hatching; no progress by the larvae already hatched.

Beef broth. Tube (No. 1), 5 hatched out of 7.

„ (No. 2), 6 „ „ 7.

„ (No. 3), 4 „ „ 7.

All the larvae were making slow progress.

Peptone broth. Tube (No. 1), 5 hatched out of 6 (2 larvae dead); slight progress.

„ (No. 2), 4 „ „ 6; slight progress.

„ (No. 3), 2 „ „ 5; very slight progress.

„ (No. 4), 2 „ „ 6; little, if any, progress.

On the 8th day from start:

Boiled distilled water. Larva still in first instar.

Peptone water. No change, the larvae still in first instar.

Beef broth. A few more eggs had hatched; the numbers are now:

Tube (No. 1), 6 hatched out of 7.

„ (No. 2), 7 „ „ 7.

„ (No. 3), 4 „ „ 7.

All the larvae show slight growth, most of them being in the second instar, but one has not passed its first moult.

Peptone broth. The living larvae are now:

Tube (No. 1), 4 out of 6 eggs.

„ (No. 2), 4 „ 6 „

„ (No. 3), 2 „ 5 „

„ (No. 4), 4 „ 6 „

Slight growth was apparent, but none had passed the first moult.

On the 11th day from start:

One of the *peptone water* tubes in which none of the eggs had hatched was inoculated with *B. coli*; by next morning (18 hours later) all the eggs in this tube had hatched.

In tube No. 4 of the *peptone broth* tubes sterility had broken down, and there was a slight bacterial infection. In this tube only were the larvae making any progress; in all the other peptone tubes the larvae were marking time.

Beef broth. In two tubes (Nos. 1 and 2), the larvae were growing steadily, if slowly; in No. 3 progress was very slow.

On the 14th day:

The larva which hatched in the boiled distilled water died in its first instar.

Peptone water. One of the two larvae which hatched in the sterile tube was dead. In the *B. coli* infected tube the larvae had already passed the second moult (about 48 hours after hatching). In the third tube, which remained sterile, the eggs were still unhatched.

Peptone broth. Tube (No. 3), one of the two larvae was dead, the other had moulted; (No. 2), 3 larvae were in the second and 1 in its third instar, all small and ill-grown. In tube No. 1 another larva had hatched, but one of those which had emerged previously was dead; of the living 2 were in the second and 2 in the first instar. In the infected tube (No. 4) the larvae were making slow but steady progress in their second and third instars.

Beef broth. Tubes Nos. 1 and 2 had become infected and the larvae were progressing.

15th day:

Distilled water. A second larva had emerged in this tube.

Tap water. Eggs still dormant.

Peptone water. In the *B. coli* infected tube all the larvae had reached their fourth instar and one had died.

To the sterile tube in which no eggs had hatched .5 c.c. of sterile watery extract of brewers' yeast was added. (Without result.)

Peptone broth. Tube (No. 1), the two first instar larvae were taken to test if change to a different media would affect growth. A note will be found at the end of this experiment (page 511), both the second instar larvae were dead; (Nos. 2 and 3), no change; (No. 4), the infected tube, the larvae were making slow progress.

Beef broth. The larvae in the infected tubes (Nos. 1 and 2) progress steadily; in No. 3 the progress is scarcely perceptible.

19th day:

Distilled water. The second larva which hatched was also dead.

Tap water. None of the eggs had hatched.

Peptone water. In the tube infected with *B. coli* all the larvae reached full growth and then died.

Of the larvae which hatched out in the sterile tube, 1 had died; the living ones made no progress.

The eggs in the tube to which sterile yeast extract was added had not hatched.

Peptone broth. (No. 1), no living larvae remained; (Nos. 2 and 3), the larvae all dead; (No. 4), growth was variable, the larvae which hatched prior to infection were ahead of the others.

Beef broth. Larvae in the sterile tube were making very slow progress; in the infected tubes they were growing steadily, though not rapidly.

22nd day:

Distilled water and Tap water. No change.

Peptone water. In the sterile tube the larvae made no progress. The yeast extract did not cause the eggs to hatch in the third tube; this tube was inoculated with a living yeast and the eggs hatched during the night.

24th day:

Tap water. The eggs lay dormant in this tube for 24 days; 1 c.c. of an autolyzed extract of brewers' yeast was added (about 20 %); the eggs hatched within 15 minutes; the larvae, however, died.

Quantities, up to about 10 %, of this sterile extract were added to the sterile tubes of peptone water and peptone broth in which unhatched eggs were lying, but no further larvae emerged, and the larvae which had already hatched in these tubes did not respond by growing.

28th day:

Peptone water tube (in which the eggs hatched on the 22nd day following an inoculation with a living yeast). The larvae showed slow and varied growth; they eventually died without pupating—the growth of yeast in the tube being presumably too feeble to supply sufficient nutrition.

The larvae in the sterile tube of peptone water to which sterile yeast extract had been added, lived on for a long while, making little or no progress. A number were still living 95 days after the start of the experiment, and one lingered on until the 113th day, when it was still in its second instar.

Beef broth. The larvae in the sterile tube progressed very slowly, sterility being maintained as shown by the sub-culture tests made at intervals. After 50 days a few adults were reared; these emerged at intervals over a period of 10 or 12 days, one, which fell into the broth and was drowned, was transferred with a platinum loop to a tube of sterile broth, but no growth ensued. A living specimen was fed on a carefully cleansed patch of skin on the arm, producing the same quickly passing reaction mark as do normally bred *S. fasciata* when they bite this individual. This observation suggests that Schaudin's opinion that the reaction following the bite of the mosquito is due to a ferment is possibly incorrect. An attempt to feed another specimen, reared subsequently under sterile conditions, on the arm of a lady, who showed a very marked reaction to the bites of *S. fasciata*, failed as the insect died without biting, although tested on several successive days.

*Result of test carried out on larvae taken from Tube No. 1
peptone broth on the 15th day.*

Two larvae were withdrawn by Pasteur pipette on the 15th day from one of the peptone broth tubes which had remained sterile.

One of these larvae was transferred to a tube of broth made from dried and powdered insects (mostly house flies), the other to a tube of water, in which horse dung had been steeped. Both these tubes had been autoclaved. The plan was to test if a change of diet under sterile conditions would affect growth. The larvae at first made no progress, but after 48 hours they began to show signs of slow growth, which continued for 30 to 40 days, by which time the larvae were little more than half grown. They then marked time during a period of 10 or 12 days and died without making any further progress.

EXPERIMENT XII.

This test was carried out with a view to deciding if sterilized media of a kind more likely to be encountered in nature than those prepared for bacteriological experiments would allow of the larvae developing.

For this purpose tubes of water in which horse dung had been steeped for 48 hours and of a broth made in hot water from dead insects were autoclaved. Both media when tested under unsterilized conditions had been found to afford excellent breeding results.

Eggs sterilized by Method *b* were transferred to the tubes by Pasteur pipette.

Insect broth. No eggs hatched within 18 hours, but a few larvae were out in tubes Nos. 1 and 2 after 48 hours. None of the eggs had hatched in tube No. 3. One or two larvae were out in this tube also, after 72 hours. It was significant, however, that a small mould was observed on a group of floating eggs in tube No. 3. (Subsequent experiments showed that the growth of a mould acted as a stimulant, causing eggs to hatch.)

During 5 days the larvae made slow but steady growth in tubes 1 and 2, but made no perceptible progress in No. 3.

Tests showed that sterility had broken down in tubes 1 and 2, a feebly growing species of bacteria being present when tested after 8 days.

The development of the insects was very slow, taking about 50 days. In tube No. 3 a larva was still living, being only in its third skin after a period of 65 days. This larva died subsequently.

There was some mortality in the late larval stage, but a few adults were reared in each of tubes 1 and 2.

Manure water. No eggs hatched within 18 hours.

A few larvae were out in one tube within 48 hours.

Larvae were out in all tubes within 72 hours.

During 5 days the larvae made slow but steady growth in all the tubes, and were in either the close of the second instar or the beginning of the third. Progress was slower in one tube than the others, but tests proved that all the tubes were infected with some feebly growing bacteria. The larvae lived some 40 or 50 days, eventually reaching the fourth larval instar, but all died without pupating.

EXPERIMENT XIII.

A number of further trials were made, using insect broth and manure water. Eggs were transferred to 12 tubes of the former and 11 of the latter; in a few of these tubes sterility was maintained and some were used in Experiment XX.

In the majority of cases the eggs hatched in consequence of the development of moulds, or showed a feeble slow-growing bacillus. The results in the sterile tubes showed that very slow or no progress took place in them. The larvae which succeeded in growing finally died before their full size was attained and there was a suspicion in these cases of some feebly growing bacillus which had escaped the sub-culture test. In the more numerous instances where the presence of the feeble infection was proved the larvae seldom completed their development.

EXPERIMENT XIV.

Use of killed culture of B. coli.

Eggs placed in tubes containing broth cultures of *B. coli*, killed by heat, failed to hatch within a period of 5 days. A larva was transferred from a sterile tube in another experiment. This larva lived for a period of over a month, but failed to make any progress. The tube was finally inoculated from a living *B. coli* culture

when the larva died. This result has followed in a number of instances when the sterile conditions in which larvae have been living for a long period are broken down. In some instances no doubt this has been due to the violent character of the growth, with which two or three small larvae are quite unable to cope, but, apart from this, it would seem that some adjustment to the sterile conditions has occurred which cannot easily be reversed. In contrast to this, is the fact that larvae which are only starved by keeping them short of food in an unsterilized condition, are quite able to take advantage of an influx of bacteria, rapid development following immediately.

EXPERIMENT XV.

Development of larvae on a diet of Staphylococcus aureus.

(Note also tube No. 7, Experiment IX.)

Eggs sterilized by Method *b* were transferred by pipette into two tubes of peptone broth. One tube was inoculated from a *B. coli* culture, the other from one of *S. aureus*.

Growth of the bacteria followed immediately in the *aureus* tube and the eggs hatched within a few hours of inoculation.

In the *B. coli* tube the inoculation failed; only 1 egg hatched, and the larvae died within 3 days. On the 4th day the tube was again inoculated from the same culture of *B. coli*; by the following morning it was observed that the second inoculation had been effective and that the eggs had hatched.

The larvae in both tubes grew rapidly and produced adults within 8 to 15 days.

EXPERIMENT XVI.

Question of the necessity of solid particles with the food, also note respecting the effect of past bacterial action on the eggs.

It was suggested by one of our colleagues that the mosquito larvae might be unable to obtain nourishment in a pure fluid, particulate matter being essential to their digestive processes. We therefore tried the effect of adding animal charcoal to some tubes and not to others. Eggs that had been kept moist from laying were used and sterilized by Method *b*. The theory here was to allow of bacterial action on the outer surface of the eggs up to the moment of their sterilization. Apparently, on the showing of this one test, the theory has some basis as the result showed more eggs hatching under sterile conditions than usual.

The eggs were transferred to tubes of peptone water, peptone broth, pure beef broth, 2% and 3% solutions of an autolyzed extract of brewers' yeast in distilled water. To the yeast extract tubes a small quantity of animal charcoal was added prior to their sterilization.

Eggs hatched in some of the tubes within an hour and many more within two hours; such rapidity suggests past rather than present bacterial action, as even if the tubes were infected at the placing of the eggs in them, there was little time for growth, whereas all the experiments suggest that it is the vigorous bacterial action of a maximum infection which counts in causing hatching.

Stegomyia fasciata

After 60 hours the following had emerged:

Peptone water	...	...	2 out of 16.	Sterile.
			0 "	6. "
Beef broth	...	...	all "	18. Infected.
			4 "	11. Sterile.
Peptone broth	...	...	1 "	17. Sterile; larva dead.
			7 "	17 "
2 % solution yeast extract	...	several ¹ .		"
			"	"
3 % solution yeast extract	...	4 tubes,	a few in each tube.	

It was noted that all the larvae in the yeast extract tubes containing charcoal were making progress, the dark line of their guts showing that the charcoal was being swallowed.

After 84 hours:

Peptone water	...	...	3 larvae from 16 eggs.	Sterile; no progress.
			0 "	6 "
Beef broth	...	...	all "	18 "
			6 "	11 "
				Rapid progress of larvae; gas production by bacterial growth.
				Very slight progress of larvae; still sterile.
Peptone broth	...	...	1 larva (dead) 17 "	Sterile.
			9 larvae from 17 "	Very slight progress of larvae; still sterile.
2 % solution yeast extract	...	4 larvae from about 15.	3 passed first moult.	
		4 "	" "	15. 2 " "
3 % solution yeast extract	...	1 "	" "	15. Has passed first moult.
		4 "	" "	15. Have " "
		3 "	" "	15. 1 has passed first moult.
		5 "	" "	15. 3 have passed first moult.

With one exception the broth and peptone tubes passed out of the experiment owing to the larvae dying after a protracted period of minimal progress, or owing to sterility breaking down in the tubes, followed by the hatching of the dormant eggs and rapid development of the larvae.

Beef broth. In one tube of beef broth sterility was maintained; on the 41st day there were 4 living larvae in the third instar; on the 76th day tests showed that the tube was still sterile, some of the larvae having advanced to the fourth instar. On the 90th day 2 adults were reared; a small quantity of broth from the tube, together with one of the adult mosquitoes, was introduced into a tube of peptone broth which remained sterile. The original tube was infected with *B. coli* to see if any eggs were lying dormant; none hatched, so a few unsterilized eggs were added to test the nutritive properties of the broth after infection. Larvae emerged from the eggs the same day, by the 8th day nearly all had passed the third moult.

Yeast extract solution. Eggs were transferred after sterilization to a few tubes of 3 % solution of an autolyzed extract of brewers' yeast; no charcoal being added,

¹ The presence of animal charcoal in the tubes rendered an exact count impossible.

larvae hatched in some of these tubes, but made still slower progress than did those with charcoal, behaving as did the larvae in sterile peptone water or peptone broth, generally dying in their first instar and but very exceptionally surviving to the third.

2 % solution of yeast extract with charcoal added. After 50 days in one tube (No. 30) there were several larvae in the third instar and at least one in the fourth; 1 had pupated but the pupa died. All the larvae were lank and slender and had failed to develop the fat bodies which, towards the close of the larval life, become rather obvious owing to the opaque white appearance that they give to the larvae.

The second tube (No. 31) contained 4 larvae in their fourth instar, all deficient in fat deposits.

One tube (No. 32) contained a single weakly looking fourth instar larva; the remainder were dead.

Another (No. 33) contained 4 fourth instar larvae, all deficient in fat.

In tube No. 34 none of the larvae had got past the third skin. By the 56th day the larvae in tubes 31 and 32 were dead.

One larva was living in tube 30 on the 76th day, but died before the 95th day. In tube No. 33 two larvae lived until the 76th day and one until the 123rd day, when it died. In tube No. 34 two larvae lived until the 76th day; between the 76th and 95th days several larvae hatched from dormant eggs in this tube. Tests showed that sterility was still maintained; by the 107th day all the recently emerged larvae were dead except one, and this had passed the first moult; two died between the 107th and 123rd day. Between the 123rd and 143rd day mounds developed in the tube and the last surviving larva died.

EXPERIMENT XVII.

A further series of tubes containing a 3 % solution of autolyzed extract of brewers' yeast in distilled water, some with and others without charcoal, were prepared.

23 days from start.

No. 51, sterile, larvae.	Slender in second or third instar.
No. 52 " "	Slender in third or fourth instar.
No. 53 " "	Great disparity of growth in all instars from first to fourth skin; all slender.
No. 54 " "	Slender in second or third instar.
No. 55 " "	Great disparity of growth in all instars from first to fourth skin; all slender.

Tubes, with charcoal, 56 and 57.

No. 56, a mould grew, the larvae had grown steadily and were in fourth instar.

No. 57, sterile larvae show slow but healthy growth, are rather more robust than those in the tubes without charcoal, but are only in second and third skin.

40 days from start.

Nos. 51 to 55 without charcoal. No appreciable progress in the last 17 days.

Nos. 56 and 57 with charcoal.

No. 56, larvae have all died without pupating.

No. 57, no appreciable progress.

56 *days from start.*

Nos. 51 to 55 *without charcoal.*

No. 51, sterile. Most of the larvae in their third instar; one or two possibly dwarfed in fourth instar.

No. 52, sterile. Only 2 larvae living, 1 in third instar, 1 in fourth instar.

No. 53, a mould present; 1 ♀ reared, larvae in second, third and fourth instars, still living.

No. 54, sterile. All in third instar.

No. 55, sterile. 2 larvae in fourth instar; 1 pupa.

With charcoal.

No. 57, all the larvae now in third instar.

78 *days from start.*

Without charcoal.

No. 51, apparently sterile; little if any change.

No. 52, " " " "

No. 53, mould present; most are in fourth instar.

No. 54, apparently sterile; little if any change.

No. 55, apparently sterile; 1 adult, 1 third or dwarfed fourth instar larva; 1 larva dead.

With charcoal.

No. 57, sterile; slight if any change.

90 *days from start.*

Without charcoal.

No. 51, sterile; no noticeable change.

No. 52, " " "

No. 53, mould has increased in size; only 1 larva living

No. 54, sterile; no noticeable change.

No. 55, " " "

With charcoal.

No. 57, sterile; slight progress.

106 *days from start.*

Without charcoal.

No. 51, sterile; no apparent change.

No. 52, sterile; dormant eggs have hatched; there are now 5 living larvae.

No. 53, all dead, the last surviving larva died on or about the 95th day.

No. 54, sterile; no noticeable change.

No. 55, " " "

With charcoal.

No. 57, sterile; only 1 larva now living.

126 *days from start.*

Without charcoal.

No. 51, sterile; little if any change; 3 larvae still living.

No. 52, sterile; 4 larvae living; those recently hatched have made slight progress.

No. 54, sterile; 2 larvae living; little if any change.

No. 55, sterile; 2 larvae living; 1 in fourth and 1 in second instar.

With charcoal.

No. 57, the last larva is now dead.

Evaporation had so reduced the quantity of fluid that boiled distilled water was added to tubes Nos. 51, 52, 54 and 55. Following the addition of the water a few eggs that had lain dormant in tubes Nos. 51, 54 and 55 hatched.

Nine days later, 135 days from start.

No. 51, it was found that all the larvae were dead.

No. 52, 1 of the 4 larvae died.

No. 54, only 1 of the 2 original larvae living; those that hatched out recently are dead.

No. 55, the recently hatched larvae have disappeared, possibly swallowed by the larger ones.

Tube No. 54 inoculated with a yeast (*S. cerevisiae*).

142 days from start.

No. 52, all the old larvae are dead, the last two died with their jaws entangled in a small tuft of hair. The three survivors are growing steadily. (Sterile.)

No. 54, this tube was inoculated with a yeast (*S. cerevisiae*); the surviving larva after one week looks large and stout in comparison with its dead companions.

No. 55, the surviving old larva is still active, two of those which hatched after the addition of fresh water are living and are now in second skin. (Sterile.)

152 days from start.

No. 52, moulds grew in the tube; the three last larvae died.

No. 54, the surviving larva pupated.

No. 55, the old larva is dead and also one of the recently hatched ones.

Tube No. 55 was inoculated with (*S. cerevisiae*).

168 days from start.

No. 54, the pupa died.

No. 55, an adult ♀ was reared from the surviving larva in this tube.

EXPERIMENT XVIII.

Killed B. coli culture and manipulated milk.

A tube of distilled water, to which washed *B. coli* had been added, was sterilized by heat, and a sterile larva introduced that had hatched out in peptone broth. After 13 days during which the larva made no progress, 1 c.c. of sterilized milk, the colloid particles of which were increased in size but not precipitated by a manipulation of its acidity, was added. No progress occurred within 3 days; the tube was inoculated from a tube of broth in which a certain species of bacterium had developed. (This culture was used because its speed of growth under the conditions of the experiment had allowed the larva in the tube to develop satisfactorily.) The larva at once commenced to grow, and pupated within 20 days, an adult emerging in due course.

EXPERIMENT XIX.

A number of tubes were prepared and sterilized containing appropriate quantities of the following in distilled water; sterile filtrate from a culture of *B. coli* with white of egg, treated so as to form a fine precipitate; filtrate of *B. coli* with milk in which the casein particles had been increased in size; and *B. coli* filtrate without any addition.

Larvae were taken from a sterile tube of peptone water in which they had hatched 16 days previously; they were still in the first instar, having made barely perceptible progress during 16 days. Slight progress followed over a protracted period in the tubes (containing white of egg and milk) which remained sterile, but in all cases it came to a standstill when the larvae were still small, in the second or third skin, after which they pined away and died. In one or two instances sterility broke down and was generally followed by a sudden spurt of growth. In most cases, however, the growth of bacteria proved over-violent and killed the insects ere they had time to develop.

EXPERIMENT XX.

The treated milk was also added to tubes of yeast extract solution, peptone water and manure water, in which eggs had hatched and the larvae were unable to develop owing to the sterile conditions. In no case did the addition of the milk produce any change in the scarcely perceptible progress that the larvae made. Sterile filtrate of *B. coli* in the proportion of about 1 in 10 was added to these tubes after the lapse of 10 or 12 days, and also to sterile tubes of insect broth and 3 % yeast extract solution. During 5 days no change resulted from the addition of the filtrate, the scarcely perceptible growth that was taking place in some only of the tubes was not appreciably quickened, in other cases when the larvae had already arrived at the marking time phase no progress was induced.

The following notes refer to some of the tubes mentioned above.

Insect broth. A larva that had hatched under sterile conditions (see Experiment XIII) lived in the tube for 34 days when it was still in the first skin; 1 in 7 of *B. coli* filtrate was added. No change took place in 5 days; then the tube was inoculated with *B. coli*; eggs which had been lying dormant in the tube for 39 days hatched as a result. Rapid growth of the larvae followed, but it is not possible to say definitely if the larva which had been marking time for so long was one of those which developed to adult stage within 10 days; 4 had pupated and 3 ♂♂ and 1 ♀ emerged in due course.

Peptone water. The two or three larvae which hatched from the eggs pipetted into the tube made no progress during 18 days; the treated milk was added—the larvae made no progress and died out within three weeks. Eggs had been lying dormant in this tube for 39 days. The tube was inoculated with *B. coli* and these eggs hatched within a few hours. The larvae commenced to make rapid progress, but their history was not followed up.

Yeast extract in distilled water. Three or four larvae hatched from the eggs placed in this tube. They made no perceptible progress during a week; treated milk was added to the tube. After 20 days two of the larvae had passed the first moult; 76 days after the addition of the milk the larvae were still in the second or

third instar, and one had failed to complete its moult satisfactorily. Three larvae were still living on the 88th day after the addition of the milk; two were living in the same stage after 104 days and one after 124 days. Sterile water was then added to the tube to replace evaporation and the tube was inoculated from a *B. coli* culture; the violence of the growth, however, killed the larvae. (Eggs were added to the tube; they hatched almost immediately and the larvae were killed off within a day, showing that the death of the larvae which had lingered on for so long a time was not in all probability connected with their age.)

Various egg hatching and larva feeding tests.

EXPERIMENT XXI.

Sterile tubes prepared of filtrate from a *B. coli* culture; *B. coli* filtrate and precipitated white of egg; *B. coli* filtrate and milk in which the casein particles had been enlarged; autolyzed yeast extract in distilled water; peptone water; and distilled water.

Eggs sterilized by Method *b* were pipetted into each tube.

No. of eggs hatched in	Tubes	2½ hours	20 hours	48 hours	96 hours
Filtrate of <i>B. coli</i> cul- ture	1	2	3	All hatched*	All* larvae growing rapidly
	1	1	1	All hatched*	All* larvae growing rapidly
	1	0	1	All hatched*	All* larvae growing rapidly
	1	0	0	1	All* larvae growing rapidly
	1	0	0	0	2, hatching became general and a mould appeared
	1	0	0*	Used for special test, see Experiment IV, page 503.	
Filtrate of <i>B. coli</i> and precipitated white of egg	1	0	1	All hatched*	Larvae growing rapidly
	1	0	0	All hatched*	Larvae growing rapidly
Filtrate of <i>B. coli</i> and treated milk	1	0	1	All hatched*	Larvae growing rapidly
Autolyzed extract of brewers' yeast, in dis- tilled water	1	0	0	2	No change
	1	0	0	0	
	1	0	0	0	
Peptone water	1	1	1	1	No change
	1	0	0	0	1
Distilled water	1	1	5	6	10
	1	1	3	3	5
	1	0	2	Used in a special test; see Experiment IV, page 503.	
	1	0	2		
	1	0	2		
	1	0	1		

* Tested for sterility—all proved to be infected.

After 120 hours. The larvae in all the starred (infected) tubes continued to grow rapidly: in the filtrate tube in which a mould appeared, all the eggs hatched but the larvae made no progress.

Films from the filtrate tubes in which sterility broke down stained by Gram's method showed a mixed infection of bacteria other than *B. coli*. One tube showed sporing forms. The results were consistent with the explanation that the tubes were infected by organisms on the eggs, some of which had escaped the sterilizing process.

Of the two peptone water tubes all the eggs in one tube hatched; a mould was noticed developing in the tube. The larvae made little if any progress during a period of 20 days, the mould gradually increasing at the surface of the fluid. The larvae were all dead within a month of hatching. 1 c.c. of the sterile filtrate of *B. coli* was added to the remaining peptone water tube and the two tubes of distilled water. Within 4 days all the eggs hatched in the peptone water tube, but it was evident that sterility had broken down. The larvae made rapid progress, but failed in their race with the bacteria—the fluid in the tube becoming very foul—and the larvae died.

Sterility also broke down in the two tubes of distilled water, to which the filtrate was added. It is quite possible that it had broken down at the time when the eggs began to hatch, but as the tube was not tested the fact was not apparent until the filtrate was added. The larvae made rapid progress at first, but it soon appeared that they had exhausted the food supply as, although they continued healthy, no progress was made. The larvae gradually died off—a few survivors lingered on until the 88th day; they were then small and undersized in the third or fourth instar. Moulds were developing in both tubes and the death of the larvae followed in a few days. Concerning the filtrate tubes to which precipitated white of egg and treated milk were added nothing of interest remains to be said; all were infected with a species of bacterium that required cool conditions—60° to 65° F.—for its favourable development. The larvae grew rapidly at first but slowed down after a few days; after the 8th day they were still in the third instar. Those in the tube to which milk was added attained the fourth instar by the 12th day and then died. The larvae in one of the white of egg and filtrate tubes developed slowly and 2 adults were reared within a month, but a mould had developed in the tube, and the remaining larva died. In the other tube a mould also developed and all died as larvae.

3% Yeast extract solution. Nos. 106 and 107. No larvae hatched within 96 hours, but one was out in each tube within 120 hours; these larvae died within 2 or 3 days. In one tube no more eggs hatched within 10 days; in the other a few larvae emerged and died. On the 12th day both tubes were inoculated from a culture of a yeast; the eggs hatched during the night, in one tube they all died, but in the other a few of the larvae made rapid progress. Three ♂ specimens were reared within 18 days of the inoculation and three ♀♀ followed a week or so later.

2% Yeast extract solution with charcoal. One tube; within 24 hours 6 or 7 larvae had hatched, the larvae making slight if any progress within 3 days of hatching; within the next few days moulds commenced to grow. By the 7th day after hatching a few larvae had passed their first moult. Some of the larvae attained to the fourth instar within 20 days of hatching, but the growth of moulds had made

considerable progress by this time, and the larvae died within 2 or 3 days. *Infection by bacteria in addition to the moulds was probable.*

EXPERIMENT XXII.

Planned to contrast the effect, if any, on hatching of *B. coli* filtrate, beef broth and distilled water. It failed owing to the number of tubes in which sterility broke down. The experiment, however, affords evidence relative to the effect of the growth of moulds and the ability of the larvae to develop on a diet of living yeast cells.

To each of 9 tubes containing 6 c.c. of sterile distilled water, was added 1 c.c. of filtrate from a culture of *B. coli*.

To each of 6 tubes was added 1 c.c. of plain beef broth.

Eight tubes contained the distilled water only.

Eggs were sterilized by Method *c* and from 12 to 20 pipetted into each tube.

After 24 hours.

Filtrate and water. 7 of the tubes have at least 20 eggs; 2 have only 12 in them. In all the tubes there are one or more larvae swimming. In most of the tubes there are 5 or 6 larvae; in one 8 or 9, and in one a dozen.

Beef broth. Four of the tubes had 12 to 18 eggs; two had at least 20 eggs in them. The hatchings were: 2:1:2:1:8:2.

Distilled water. Six of the tubes had from 12 to 20 eggs; two had 20. The hatchings were: 1:1:0:0:0:2:0:0.

After 4 days.

Filtrate and water. In one tube (No. 9) there was a bacterial growth; the water was cloudy; all the eggs had hatched and the larvae showed vigorous progress. In all the remaining tubes, though hatching was general, larval progress was not evident; there was no bacterial growth, but moulds had commenced to grow in the tubes.

Beef broth and water. In two tubes (2 and 6) bacterial growth started; in two (1 and 5) moulds grew, while two (3 and 4) remained sterile. Hatching was general, but not complete in those tubes which were not infected by bacteria or moulds; larval growth was rapid only in those containing bacteria.

Distilled water. In two of the tubes two or three eggs hatched; in three one hatched, while in three no larvae emerged.

Subsequent history.

Filtrate and water. Nine tubes numbered 1 to 9.

No. 1 had a double infection of bacteria and moulds; one undersized ♂ bred after 35 or 40 days, remainder died.

No. 2, white surface moulds gradually increased, and the larvae gradually died off; 1 lived 60 days, and died in the third instar.

No. 3, dark surface moulds grew gradually; 1 larva lived 53 days but died in the third instar.

No. 4, grey surface moulds grew gradually; none of the larvae passed the second moult—all died within 50 days.

No. 5, the mould in this tube was more vigorous; it formed flocculent tufts just beneath the surface. The last larva to die was in its third instar. None lived beyond 50 days.

No. 6, white surface moulds grew gradually; 3 larvae were living on the 53rd day; the last survivor died in the third instar within 60 days.

No. 7, in addition to floating moulds, this tube had a heavy bacterial growth in it. 2 fourth instar and 1 second instar larvae were living on the 69th day, but all died without reaching pupal stage.

No. 8, small globular moulds developed at the bottom of the tube; all the larvae died in the first or second instar early in the course of the experiment.

No. 9, this tube had an obvious infection, a surface scum formed, but there was no trace of masses of mould, as in the other tubes; 5 larvae lingered in the second and third instars until the 53rd day, but all died out by the 69th.

Beef broth and water.

No. 1, a white surface mould; all larvae died in first and second skin.

No. 2, a weak, double bacterial infection; larval progress slight; several larvae in second and third skin were living on 53rd day; by the 106th day 1 larva was still living and a sub-culture in broth gave no result, the infection being presumably too slight. 1 c.c. of broth was added to the tube and subsequently .5 c.c. from the tube was added to sub-culture broth tube. Result: infected. A larva hatched from a dormant egg within two hours of the addition of broth, presumably as the result of increased bacterial growth consequent upon the addition of fresh nutritive material.

No. 3, this tube remained sterile to all the tests applied; 1 larva reached second and 1 the third skin by the 53rd day; both died subsequently.

No. 4, this tube remained sterile; 5 larvae were still living in the first instar on the 21st day; on the 28th day the larvae showed little if any progress. A mass of living yeast cells (*S. cerevisiae*), lifted from a wort agar slope, was added to the tube; several dormant eggs hatched within a few minutes. The larvae all grew rapidly and cleared the water, which became cloudy when the yeast cells were introduced, within 5 days. A fresh loopful of the yeast cells was added. In 2 days' time the water was again clear, and a fresh loopful was given. On the 13th day most of the larvae were in their fourth skin and one had pupated. Another loopful of the yeast cells was given. A week later a male was reared and a loopful of yeast cells added. A second adult, a ♀, was reared on the 25th day after infection. A further loopful of the yeast cells was given; by the 32nd day 3 adults had been reared. There were 2 pupae and 4 fully developed larvae; further yeast cells were given. In all 8 adults were reared in 40 days. The period taken in this instance was due to the lapse of time between the meals and the small allowance of food given at any one time; had a larger mass been given, not above one-third of the time would have been taken.

No. 5, several very small moulds grew at the bottom of the tube. The larvae made slight progress; the moulds did not develop, and apparently died out. The larvae made very slight progress; but by the 40th day after hatching none had passed the second instar. Some of the larvae died, others made slow progress. After 100 days there were 2 living, 1 in the third and 1 in the fourth instar. Tested and found sterile on the 100th day.

No. 6, this tube developed a vigorous bacterial growth; the larvae made rapid progress, but all died in the second or third skin.

Distilled water. Of the eight tubes to which eggs were added only one showed infection by the tests used; on the 20th day of the experiment the position was as follows:

No. 1, 2 eggs hatched, 1 larva dead, 1 living, both in first skin.

No. 2, none hatched.

No. 3, 3 hatched, 1 larva dead, 2 living, all in first skin.

No. 4, 6 hatched, all dead in first skin.

No. 5, none hatched.

No. 6, 5 hatched, 4 dead, 1 living, all in first skin.

No. 7, many hatched, probably all, larvae all dead but one; none past first skin, infected by mould.

No. 8, 1 hatched; larva living in its first skin.

All the living larvae died within a week or two; the tubes containing dormant eggs were used for the under-noted experiment.

On the 28th day of experiment a loopful of living yeast cells (*S. cerevisiae*) was added to tube No. 1. Eggs which had been lying dormant for 28 days hatched within 5 minutes; rapid growth ensued and from time to time a fresh loopful of the yeast cells was added. 5 adults were reared and one specimen died in pupal stage. The time taken was from 30 to 50 days; the quantity of food given at five-day or weekly intervals—was a small mass of cells scraped from a wort agar slope with a platinum loop—only allowing of development by fits and starts.

Tubes Nos. 2, 3, 4, 5, 6 and 8 were infected by adding .5 c.c. of a yeast culture; larvae hatched from dormant eggs in most of the tubes within 5 minutes; others followed rapidly.

All the larvae made rapid progress except those in tube No. 5; here moulds started to grow rapidly. Within a week there was only one larva living, and this one also died within a day or two.

A small quantity, usually about .2 or .3 c.c. of the yeast culture, was added at intervals of several days or a week; the point it was wished to establish being not the speed with which the larvae could develop on a diet of yeast cells, but the fact that they marked time during the intervals and spurted when the food was given. In spite of the rather crowded condition of the larvae in 6 or 7 c.c. of fluid, they remained healthy, except in tube No. 4 where moulds developed. In this tube all the larvae died. The result showed as anticipated the entire dependence of the larvae on the added yeast culture, their speed of progress being conditioned by the length of the periods between each addition to the tubes. In tube No. 2, 6 adults were reared, in tube No. 3, 6, and 6 individuals in tube No. 6; while in tube No. 8 which contained more larvae to start with 9 adults were reared.

EXPERIMENT XXIII.

The increasing proportions of tubes in which sterilization broke down either from sporing species of bacteria or moulds, suggested that the contamination of the eggs laid in the breeding-pan was very gross. Steps were therefore taken to obtain eggs laid under more cleanly conditions and less likely to be heavily infected with sporing bacteria and moulds (the method adopted is described on pp. 488-9).

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Eggs were used that had had time to incubate, but had not dried; these were well washed in three changes of clean tap water, put on to clean filter paper and then dried; they were subsequently sterilized by washing in weak .5% lysol, then in 2%, then in 1%, and then in sterilized distilled water; 9 or 10 eggs were transferred by Pasteur pipette to the following tubes:

Distilled water, Nos. 1 to 6.

Distilled water with 1 c.c. of sterile *B. coli* filtrate (= 1 in 6), Nos. 7 to 12.

Beef broth (without peptone or salt), Nos. 13 to 18.

Beef broth with 1 c.c. of sterile *B. coli* filtrate (= 1 in 6), Nos. 19 to 24.

Media	No. of tube	Hatching after 24 hours	After 5 days	Remarks after 5 days
Distilled water	1	0	0	The single larva which emerged did not grow. In one or two other tubes eggs uncapped, but the larvae died without quitting the shell.
	2	0	0	
	3	1	1	
	4	0	0	
	5	0	0	
	6	0	0	
Distilled water and filtrate of <i>B. coli</i> (= D.W. 6 c.c., F. 1 c.c.)	7	0	0	Sterility broke down after 24 hours in Nos. 9, 10 and 11, and the larvae which emerged made rapid progress.
	8	0	0	
	9	2	All*	
	10	0	All*	
	11	3	All*	
	12	0	3	
Beef broth without salt or peptone	13	2	All*	Heavy growth of bacteria, larvae already in fourth instar. Larva makes no progress. Larva makes scarcely any progress. Larva makes no progress. Larva makes slight progress.
	14	1	1	
	15	0	1	
	16	0	1	
	17	0	0	
	18	1	1	
Beef broth with filtrate of <i>B. coli</i> added (= B.B. 6 c.c., F. 1 c.c.)	19	0	0	Larvae show no progress. Larva shows no progress. Heavy bacterial growth, larvae are already in fourth instar; remaining eggs failed to hatch. Larva shows no progress.
	20	0	2	
	21	0	1	
	22	0	7*	
	23	0	0	
	24	1	1	

On the 6th day tube No. 15 was inoculated from No. 13; all the eggs had hatched by the following morning.

After the 7th day.

Distilled water and Filtrate. There was a distinct difference as regards the progress of the larvae in the infected tubes 9, 10 and 11; while the larvae in 9 and 11 were on a parity as to size, those in 10 were slightly but definitely smaller. Sub-

* Infected tubes.

cultures from these tubes show that, while the bacteria infecting 9 and 11 are closely allied, probably the same species, the infection of 10 differs in that it shows a much less vigorously growing species. The interesting point is that, although the difference in larval progress is slight, the divergence between the vigour of bacterial growth is extreme.

After the 13th day.

Distilled water. Tubes 1 to 6. The only larva which emerged has made no progress and is now very feeble.

Distilled water and Filtrate. The larvae in tubes 9, 10 and 11 are all of full size, but none of them have as yet pupated. In No. 12 three more eggs have hatched, none of the 6 larvae have as yet passed their first moult.

Beef broth. In tube No. 13 all the larvae have been killed by the vigour of the bacterial growth. In No. 14, 3 more eggs have hatched. In No. 15 the larvae are now either just approaching or past the second moult. This tube was passed on to a friend and kept at a lower temperature than the others—60° F. instead of 75° F. In No. 16 another egg has hatched, neither of the two has made any progress and one has died. In No. 17 one egg has hatched. In No. 18, 5 more eggs have hatched, but only the larva which emerged 12 days ago has made any progress and this one has not yet moulted.

Beef broth and Filtrate. No. 20, one of the two larvae is dead; their progress since hatching is only just perceptible.

No. 21, the larva has died without moulting.

No. 22 (infected), 7 adults have been bred, 6 ♂♂ and 1 ♀; 1 living pupa remains.

No. 23, 1 egg has hatched.

No. 24, the larva has died without moulting.

After the 21st day.

Distilled water. 1 to 6; the larva which emerged in tube 3 died in its first instar.

Distilled water and Filtrate. In tubes 9 and 10 the larvae are marking time in their fourth instar. In No. 11, 2 ♂♂ have been bred, the remaining larvae, of full length, are marking time. In No. 12 the 6 larvae make scarcely perceptible progress.

Beef broth. In tubes Nos. 14, 16, 17 and 18 the larvae are making very slow progress; a few are approaching, and one or two have passed their first moult. In tube No. 15, inoculated from No. 13 and passed on to a friend, the larvae made comparatively slow progress, presumably owing to the low temperature at which the tube was kept; apparently the vigour of the bacterial growth was the cause of their destruction.

Beef broth and Filtrate. In tube No. 19 one egg uncapped, but the larva was either already dead or died immediately afterwards, as it failed to get clear of the eggshell.

No. 20, 2 more eggs have hatched. The survivor of the first two larvae is now in its second instar.

No. 22 (infected), another adult was reared (8 in all).

No. 23, the larva from the only egg which hatched is now in its second instar.

No. 24, another egg has hatched; the larva is now in its second instar.

After 28 days.

Distilled water. No change; eggs still dormant.

Distilled water and Filtrate. Nos. 9 and 10, the larvae still marking time.

No. 11, another adult reared; some of the larvae have died, only 1 is now living.

No. 12, only 4 of the 6 larvae are living; all the larvae are still in their first instar.

Tubes Nos. 7 and 8 were used in a demonstration at a meeting of the Entomological Society of London. To No. 7 was added a small mass of yeast cells from a wort agar culture of *S. cerevisiae* and to No. 8 a small mass from a culture of *B. coli*. The eggs in No. 7 hatched within a few minutes, those in No. 8 during the following night.

Beef broth. No. 14, 1 larva in first and 3 in the second instar.

No. 16, 3 larvae in first and 2 in second instar.

No. 17, 4 larvae in first and 1 in the second instar.

No. 18, 1 larva has died; 4 are still in their first and 1 is in its second instar.

Beef broth and Filtrate. No. 19, no change.

No. 20, another egg has hatched; 3 larvae are in their first and 1 in its third instar.

No. 21, no change.

No. 23, another egg has hatched; the first larva is still in its second instar.

No. 24, 2 more eggs have hatched; the older larva is now in its third instar.

After the 33rd day.

Distilled water. No change.

Distilled water and Filtrate. Nos. 7 and 8, there is a marked difference in the growth of the larvae in these two tubes. Approximately the same bulk of *B. coli* was put into No. 8 as there was of yeast cells into No. 7. The larvae in No. 7 have only reached the second and third instars while those in No. 8 are all either in the third or fourth instar. This difference can hardly be owing to a minute difference in mass of the bacteria or yeasts which were added; probably it is due to the medium (distilled water with a little filtrate of a broth culture of *B. coli* added) being better adapted for the multiplication of bacteria than the yeasts. The fourth instar larvae in tubes 9 and 10 and the remaining larva in tube 11 are all marking time, having presumably exhausted all the nutriment without fully developing their fat bodies. In tube 12 all the larvae, save one, died in their first or second instars; the survivor, still in its first instar, is only capable of feeble movement.

Beef broth. No. 14, 1 larva is dead.

No. 16, no noticeable change.

No. 17, two larvae have died, the survivors are 1 in second and 1 in third instar.

No. 18, no change.

Beef broth and Filtrate. Nos. 19 and 20, no change.

No. 21, the larva which was in its second instar is dead.

No. 23, no change.

No. 24, only 1 larva in its first instar is now living.

After 49 days.

Distilled water. No change.

Distilled water and Filtrate. No. 7, these larvae are now in their third and fourth instars.

No. 8, 4 adults have been reared (within 21 days); there still remain 1 pupa and 1 full-grown larva in the tube.

No. 9, all the larvae but one are dead.

No. 10, 1 adult has been reared; there are still 4 full-grown larvae in the tube.

No. 12, the only larva is dead.

Beef broth. No. 14, no change.

No. 16, 1 has died; the 3 survivors have made no appreciable progress.

No. 17, the larva in its second instar has died, but 2 more eggs have hatched; the remaining larva is still in its third instar.

No. 18, 2 of the larvae in first instar have died.

Beef broth and Filtrate. No. 19, no change.

No. 20, 1 of the larvae has died.

Nos. 21, 23 and 24, no change.

After 50 days.

Beef broth and Filtrate. Tube No. 19 was inoculated from a culture of *B. coli* on the 50th day; 8 or 9 eggs hatched within a few hours.

After 60 days.

Beef broth and Filtrate. About .5 c.c. of a broth culture of *B. coli* was added to tube No. 21 on the 60th day. A number of dormant eggs hatched within 10 to 15 minutes.

After 69 days.

Distilled water. No change.

Distilled water and Filtrate. No. 7, 1 adult reared; 1 died in pupal stage; 3 fourth instar larvae still living. A slow growing surface mould is present.

No. 8, 2 more adults reared (6 in all).

Nos. 9, 10 and 12, no change.

Beef broth. No. 14, another larva has died; the 2 survivors appear very feeble.

No. 16, all the larvae are dead.

No. 17, 1 of the 3 larvae is dead.

No. 18, 1 of the 2 larvae is dead.

Beef broth and Filtrate. No. 19, 5 adults reared, 1 larva died when full grown.

(Note these eggs were dormant for 50 days and the adults were reared within 20 days of inoculation of the tube with *B. coli*.)

No. 20, 1 larva has died.

No. 21, the larvae which hatched in this tube after its inoculation with *B. coli* were full grown within 7 days.

No. 23, the second instar larva is dead; another egg has hatched.

No. 24, no change.

After 78 days.

Distilled water. No. 7, a second adult reared; 1 larva living, the others died in fourth instar.

No. 9, the last larva has died.

No. 10, a second adult reared; 3 living larvae remain.

No. 12, no change.

Beef broth. No. 14, both the larvae are dead.

No. 17, both the larvae are dead.

No. 18, the larva is dead.

Beef broth and Filtrate. No. 20, no change.

No. 21, 7 adults reared within 16 days of the hatching of the eggs.

No. 23, no change.

No. 24, the third instar larva is dead; only 1 of the younger larvae is living, it is still in its first instar.

After 87 days.

Distilled water. No change.

Distilled water and Filtrate. No. 7, 1 larva marking time; moulds are growing out of the mouths of the dead larvae.

Nos. 10 and 12, no change.

Beef broth. No change.

Beef broth and Filtrate. No. 20, only 1 first instar larva is now living.

No. 21, the last larva has died.

Nos. 23 and 24, no change.

After 100 days.

Distilled water. Tubes 4, 5 and 6 were used for the following experiment in conjunction with Experiment XXIV, page 530.

To tube No. 4 about 3 % of a living culture of *B. coli* was added.

„ No. 5 „ 3 % of a sterile autolyzed extract of brewers' yeast.

„ No. 6 „ 3 % of a killed culture (autoclaved) of *B. coli*.

Result: No. 4, 1 hatched within 2 minutes; 2 more within 5 minutes; 2 more within 10 minutes.

No. 5, 1 hatched within 15—20 minutes.

No. 6, 1 „ „ „ „

After an hour and a half:

No. 4, 8 out of 10 eggs hatched.

No. 5, 1 „ 9 „ „

No. 6, 1 „ 11 „ „

A small quantity of living yeast cells from a wort agar culture was then transferred by platinum loop to tube No. 5 and the remaining 8 eggs hatched within 15 minutes.

About 3 % of a living broth culture of *B. coli* was added to No. 6, and 1 egg hatched within 3 minutes, another followed within 5 minutes and 4 more within 15 minutes = 6 out of 10.

After 110 days.

Distilled water. Nos. 1, 2 and 3, no change.

No. 4, larvae now in second instar.

No. 5, „ „ third and fourth instars.

No. 6, „ „ second and third instars.

The rapid growth of the larvae in tube No. 5 should be noticed in contrast to what happened with regard to tubes Nos. 7 and 8, distilled water and filtrate, after infection with yeasts and bacteria on the 28th day. Presumably this difference is due to the media in tube No. 7 having been unfavourable for the yeast.

Distilled water and Filtrate. No. 7, a further development of moulds; the larva is dead.

No. 10, all died as larvae with the exception of one which reached the pupal stage.

No. 12, an egg had hatched.

Beef broth. Nos. 14, 16, 17 and 18, all the larvae in these tubes are dead; possibly some living eggs remain dormant.

Beef broth and Filtrate. Nos. 20, 23 and 24, all the larvae are dead; possibly some living eggs are lying dormant.

After 134 days.

Distilled water. Nos. 1 and 3, small white moulds developed in the tubes.

Nos. 4, 5 and 6, want of nutriment checked development; a few larvae are living in their second and third instar but many are dead.

Distilled water and Filtrate. No. 10, another adult reared.

No. 12, another egg hatched; both the larvae which hatched after the 87th day and this one which hatched after the 110th day are in their first instar.

Beef broth. No larvae are living in these tubes, but it is possible that there may be dormant eggs.

Beef broth and Filtrate. In No. 23 an egg has hatched since the 110th day.

After 156 days.

Distilled water. Tube No. 2 was infected with *B. coli* without result; it was concluded that all the remaining eggs were dead.

Nos. 4, 5 and 6, 1 larva was living in No. 4 and 3 in No. 5; all were dead in No. 6.

Distilled water and Filtrate. No. 12, only 1 larva living.

Beef broth. No. 14, tested with *B. coli* for dormant eggs; none hatched.

No. 16, an egg had hatched since the 134th day.

Nos. 17 and 18, a little of a broth culture of *B. coli* was added; dormant eggs hatched in both tubes within 15 minutes.

Beef broth and Filtrate. The larva which hatched out of a dormant egg in No. 23 is dead.

An egg hatched in tube No. 20 after the 134th day.

After 203 days.

All the larvae are dead except the one in distilled water and filtrate No. 12; this is now in its fourth instar. One adult was reared in tube No. 5, distilled water.

The remaining tubes were infected with a yeast to see if any dormant eggs survived. Single eggs hatched in tubes No. 1 distilled water and No. 6 broth and filtrate. Further quantities of yeast cells were added to distilled water and filtrate tube No. 12 on the 204th day; the larva pupated on the 205th day and an adult ♂ was reared on the 208th day.

EXPERIMENT XXIV.

Planned to test further the effect of sterile cultures of bacteria and extracts of yeasts on dormant eggs.

Twenty-four tubes each containing 8 c.c. of distilled water were sterilized and a number of sterile eggs varying from 20 to 50 were introduced. After 5 days the tubes were examined, and a selection made of those in which only a few eggs had hatched.

The following were chosen:

No. 1,	about 50 eggs,	6 hatched;	all the larvae dead.
No. 2	„ 25 „	none „	
No. 3	„ 40 „	2 „	all the larvae dead.
No. 4	„ 30 „	1 „	larva dead.
No. 5	„ 50 „	4 „	1 larva living.
No. 6	„ 45 „	3 „	all the larvae dead.
No. 7	„ 30 „	3 „	2 larvae living.
No. 8	„ 30 „	3 „	all the larvae dead.
No. 9	„ 25 „	none „	
No. 10	„ 25 „	2 „	all the larvae dead.
No. 11	„ 40 „	2 „	„ „ „
No. 12	„ 20 „	1 „	larva dead.
No. 13	„ 35 „	5 „	1 larva living.
No. 14	„ 30 „	4 „	2 larvae living.

Ten tubes were not used.

The cultures or extracts used were as follows:

Autolyzed extract of brewers' yeast, *B. coli*, *Staphylococcus aureus*, sterilized by autoclaving. *B. coli* and *S. aureus*, sterilized by steaming. *B. coli*, killed by chloroform and a sterile filtrate of *B. coli*. This last was several weeks old at the time of the experiment.

All the media had been kept for 48 hours and sub-cultured to test for sterility before use; .5 c.c. = 6 % was added to each tube except in the case of the autolyzed yeast in which test .2 c.c. = 2.5 % was added to one tube and .5 c.c. = 6 % to the other.

Autoclaved:

		No. of Tube		
Autolyzed yeast extract...	...	No. 1,	2.5 % added	} Hatching commenced within 5 minutes.
		No. 2,	6 %	
<i>B. coli</i> ...	...	No. 3,	6 %	} Hatching commenced within 5 minutes.
		No. 4,	6 %	
<i>S. aureus</i> ...	...	No. 5,	6 %	} No effect within 10 minutes.
		No. 6,	6 %	

Steam heated :

	No. of Tube		
<i>B. coli</i>	No. 7, 6 %	added	} Hatching commenced within 5 minutes.
	No. 8, 6 %	„	
<i>S. aureus</i>	No. 9, 6 %	„	} No effect within 10 minutes.
	No. 10, 6 %	„	

Killed with Chloroform :

<i>B. coli</i>	No. 11, 6 %	„	} Hatching commenced within 10 minutes.
	No. 12, 6 %	„	
Filtrate of <i>B. coli</i>	No. 13, 6 %	„	} No effect within 10 minutes.
	No. 14, 6 %	„	

*After half-an-hour.**Autoclaved cultures :*

Autolyzed yeast extract... Nos. 1 and 2, all or a high percentage hatched.

B. coli Nos. 3 and 4, „ „ „ „

S. aureus Nos. 5 and 6, „ „ „ „

Steam heated :

B. coli Nos. 7 and 8, all or a high percentage hatched.

S. aureus No. 9, only one or two had hatched.

No. 10, all or a high percentage had hatched.

Killed by chloroform :

B. coli Nos. 11 and 12, a large number but certainly not all.

Filtrate of *B. coli*... Nos. 13 and 14, no effect.

*After 18 hours.**Autoclaved :*

Autolyzed yeast extract... Nos. 1 and 2, all hatched.

B. coli Nos. 3 and 4 „

S. aureus Nos. 5 and 6 „

Steam heated :

B. coli Nos. 7 and 8 „

S. aureus Nos. 9 and 10 „

Killed by chloroform :

B. coli Nos. 11 and 12 „

Filtrate :

B. coli Nos. 13 and 14, a good proportion but by no means all hatched.

It is to be noted that considerable numbers of eggs hatched out during this period in some of the remaining 10 tubes to which no addition had been made.

After 11 days.

Notes regarding the progress of the larvae.

Autoclaved :

Autolyzed yeast extract... No. 1, infected bacterial growth; larvae in second and third instar, 2 in fourth.

No. 2, sterile, larvae still in first instar; no progress.

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				No. of Tube
<i>B. coli</i>	...	...	...	No. 3, infected, larvae still in second instar; growing.
				No. 4, sterile, larvae still in first instar; no progress.
<i>S. aureus</i>	...	...	...	No. 5, apparently sterile, larvae still in first instar; no progress.
				No. 6, apparently sterile, larvae still in first instar; but growing.
<i>Steam heated :</i>				
<i>B. coli</i>	...	...	...	No. 7, ? infected } Larvae growing in first and
				No. 8, ? infected } second instars.
<i>S. aureus</i>	...	...	...	No. 9, infected, larvae mostly in second instar, growing.
				No. 10, sterile, larvae still in first instar; no progress.
<i>Killed by chloroform :</i>				
<i>B. coli</i>	...	...	...	No. 11, infected, and a mould growing, larvae of varied growth, first to third instars.
				No. 12, sterile, larvae in first instar; no progress.
<i>Filtrate :</i>				
<i>B. coli</i>	...	...	...	No. 13, infected, all eggs hatched, larvae in second or third instars; growing.
				No. 14, sterile, a number still dormant; larvae in first instar; no progress.

Unfortunately time did not permit of sub-culture tests to clear up doubtful points as to infection of tubes.

The suggestion conveyed by the number of breakdown in sterility is that the eggs were imperfectly sterilized.

After 35 days.

Nine spare tubes of distilled water: many eggs had hatched in all these tubes. In three of them some colourless, fuzzy looking growth has occurred, probably of fungoid origin. In six there are no signs of any growth; in a tube that was infected with *B. coli* a few larvae had reached their third instar.

Autolyzed yeast extract... No. 1, not much further progress; some had died.

No. 2, 2 or 3 had reached the second instar, the rest were dead.

B. coli ... No. 3, larvae had reached the second or third instar; many were dead and moulds were growing, in many cases from the mouths of the dead larvae.

No. 4, larvae in second or third instars seemingly quite healthy; this tube is now infected.

S. aureus ... No. 5, only 2 living larvae, 1 in first and 1 in fourth instar; the latter was full grown; a surface mould was growing—probable bacterial infection also.

No. 6, only 1 third instar larva was living; a mould had almost covered the surface of the tube.

			No. of Tube	
<i>B. coli</i>	...	...	...	No. 7, 4 larvae were living—all in third instar (probably infected).
				No. 8, 3 larvae were living—all in third instar (certainly infected).
<i>S. aureus</i>	...	...	...	No. 9, 2 living larvae in third instar (certainly infected).
				No. 10, all dead in second instar (apparently still sterile).
<i>B. coli</i>	...	...	...	No. 11, all dead; at least 2 larvae gained their fourth instar.
				No. 12, all dead in second instar (apparently still sterile).
<i>B. coli</i>	...	...	...	No. 13, 4 larvae living in third instar; all the others died in this stage (probably infected).
Filtrate	...	...	...	No. 14, a number of living larvae all still in first instar (apparently still sterile).

The history of the larvae was not continued beyond this point.

As the effect of the addition of dead cultures of bacteria to the tubes in this experiment was so much at variance with previous trials and it seemed probable that the difference was due to some variation in the eggs, an endeavour was made to check these results in the following way:

A small quantity of autolyzed yeast and dead *B. coli* culture used in this experiment were therefore added to some tubes of distilled water taken from Experiment XXIII in which eggs had been lying dormant for 100 days. These tubes were numbered 4, 5 and 6. The fluids added were autolyzed yeast and *B. coli* which had been autoclaved, and a living culture of *B. coli* as control.

To tube No. 4, about 3 % of living *B. coli* culture.

„ No. 5 „ 3 % of sterile autolyzed yeast (autoclaved).

„ No. 6 „ 3 % of sterile *B. coli* culture (autoclaved).

Result:

No. 4, 1 egg hatched within 2 minutes; 2 more within 5 minutes; 2 more within 10 minutes.

No. 5, 1 egg hatched within 15—20 minutes.

No. 6, 1 egg hatched within 15—20 minutes.

After an hour and a half.

No. 4, 8 out of 10 eggs had hatched.

No. 5, 1 „ 9 „ „

No. 6, 1 „ 11 „ „

These figures include the eggs hatched within the first 20 minutes.

A small quantity of living yeast cells from a wort agar culture was then transferred by platinum loop to tube No. 5, the remaining eggs hatched within 15 minutes; all of 8.

About 3 % of a living culture of *B. coli*, in broth, was added to tube No. 6; 1 egg hatched within 3 minutes; another followed within 5 minutes and 4 more within 15 minutes = 6 out of 10.

The failure of 4 eggs to hatch was probably due to their being dead.

EXPERIMENT XXV.

Yeast and yeast extracts.

Eggs sterilized by a slight variant of Method c were transferred by pipette into tubes containing 6 c.c. sterile distilled water.

The number of eggs in each tube varied between about 25—50.

On the 16th day an examination of tubes showed:

No. 1,	3 eggs hatched.	Larvae dead.
No. 2, 3	„	„
No. 3, 5	„	„
*No. 4,	none hatched.	
No. 5, 6	eggs hatched.	Larvae dead.
No. 6, 2	„	1 larva dead and 1 living.
No. 7, 3	„	1 „ „ 2 „
*No. 8,	none hatched.	
No. 9,	1 egg hatched.	Larva dead.
No. 10,	6 eggs hatched.	5 larvae dead and 1 living.
No. 11, 10	„	9 „ „ 1 „
No. 12, 10	„	Larvae dead.

* The number of eggs in these tubes was smaller than in the others, but more than 10.

To tubes Nos. 1, 8, 3 and 7.5 c.c. (= 1 in 12) of a sterile watery extract of yeast was added.

To tubes Nos. 2, 9, 5 and 10.5 c.c. (= 1 in 12) of sterile autolyzed yeast extract was added.

To tubes Nos. 4, 6, 11, 12 a small mass of living yeast cells from a wort agar culture was added.

After 10 minutes.

Living yeasts (*S. cerevisiae*):

Numbers of eggs had hatched in tubes 6—12 and 11, but only one in tube

No. 4, but others followed almost immediately.

Watery extract of brewers' yeast:

2 hatched in No. 3, a number in No. 1, none in No. 8, and only 1 in No. 7.

Autolyzed extract of brewers' yeast:

Numbers hatched in tubes Nos. 2, 9, 5, and 10.

After 45 minutes.

Living *S. cerevisiae*:

Numbers, probably most, of the eggs had hatched in Nos. 6, 12, and 11; a few in No. 4. The numbers were too large for accurate counting, but one got the impression that a smaller proportion of eggs have hatched in No. 4 than in the other tubes.

Autolyzed extract of brewers' yeast:

Hatching was general in tubes Nos. 2, 9, 10 and 5.

Watery extract of brewers' yeast:

None had hatched in No. 8, only 2 in No. 3; a good proportion, probably one-third, had hatched in No. 1; only 3 in No. 7 out of a large number (probably quite 50 eggs).

Tube No. 10 was infected from No. 4 to test the effect on larval growth.

After 18 hours.

The hatching is now general in all the tubes to which living yeasts or autolyzed yeast extract was added.

• Living yeasts (*S. cerevisiae*):

The larvae in tubes Nos. 4, 6, 11 and 12 were distinctly in advance of those in tubes Nos. 2, 5, 9 and 10, to which autolyzed yeast extract was added.

Tube No. 10, inoculated from No. 4, showed no advantage to larval growth as yet.

Watery extract of brewers' yeast:

Tubes Nos. 1, 3, 7 and 8 do not show consistent results. In No. 1, 8 eggs had hatched—a fair proportion of the eggs present (about 22 to 25). In No. 3, only about 3 eggs have hatched out of some 20—25. In No. 7, 10 have hatched (about half the eggs). In No. 8, only 3 eggs have hatched out of 15—20.

After 36 hours.

Watery extract of brewers' yeast:

Sterility had broken down in No. 7 and hatching was general. In No. 1 no more eggs had hatched. In No. 3 hatching was general, yet there is no evidence of infection. In No. 8 only 3 eggs had hatched.

Living yeasts (*S. cerevisiae*):

In these tubes the growth of the larvae is greatest in those which contain the fewest larvae.

Autolyzed extract of brewers' yeast:

The larvae in tube No. 10 (infected) show no advantage as yet over those in Nos. 2, 5 and 9.

After 4 days.

Watery extract of brewers' yeast:

- Tube No. 1, tests show that this tube is still sterile; many eggs remain dormant.
- „ No. 7 (infected), larval progress has been rapid.
- „ No. 8, sterility broke down shortly after the previous note; all the eggs have now hatched.
- „ No. 3, hatching had been very general in this tube, but it still remains sterile.

Autolyzed extract of brewers' yeast:

- Tube No. 9, a bacterial growth was in progress and larval development was rapid.
- „ No. 2, had been infected; the larvae were progressing.
- „ No. 5, remained sterile; larvae showed scarcely any progress.
- „ No. 10 (infected), larvae showed varied progress; a few were well grown in third instar, others were still small in first or second instar.

After 18 days.

Watery extract of brewers' yeast:

- Tube No. 1 (sterile), none of the larvae had passed their first moult.
- „ No. 3 (sterile), none of the larvae had passed their first moult; all dead but three.
- „ No. 8 (infected), varied growth in second, third and fourth instar.

Tube No. 7 (infected), larval growth had slowed down; none had yet reached their fourth instar.

Autolyzed extract of brewers' yeast:

Tube No. 5 (sterile), larvae were all still in first instar.

„ No. 2 (infected), larvae in various stages of growth in second and third instar.

„ No. 9, all killed by vigour of bacterial growth.

„ No. 10 (infected), in third and fourth instar.

Living yeasts (*S. cerevisiae*):

The larvae in these tubes had been given more yeasts. In the tubes where they were most numerous they were in third and fourth instar. In tubes Nos. 11 and 12 where fewer larvae were present a few adults had already been bred and the remainder were either full grown larvae or pupae.

After 42 days.

Watery extract of brewers' yeast:

Tube No. 1 (sterile), all were dead; none past second instar.

„ No. 3 (sterile), 3 or 4 living in second instar, the rest dead in first instar.

„ No. 8 (infected), nearly all dead in various stages.

„ No. 7 (infected), all dead but one in its third instar.

Autolyzed extract of brewers' yeast:

Tube No. 5 (sterile), a few had reached their second instar.

„ No. 2 (infected), but little, if any progress since 18th day.

„ No. 10 (infected), 1 adult reared, 1 pupa, many full-fed larvae.

Living yeasts (*S. cerevisiae*). No food since 18th day:

Tube No. 4, all dead, but 1 in third instar.

„ No. 6, all dead, but 1 in third instar.

„ No. 11, 2 adults reared; remainder dead in various stages.

„ No. 12, all dead, but 1 in fourth instar.

The history of the larvae was not continued beyond this point.

REFERENCES.

- ¹ AGRAMONTE (1902). *Berkeley's Laboratory work with Mosquitoes*. New York, 1902. 112 pages, 16 figs.
- BACOT (1916). *Yellow Fever Commission (West Africa)*. Reports on Questions connected with the Investigation of non-malarial Fevers in West Africa. III. 1-191. J. and A. Churchill, London.
- BOYCE and LEWIS (1910). The effect of Mosquito larvae upon drinking water. *Ann. Trop. Med. and Parasitol.* III. 591-594.
- HOWARD, DYAR and KNAB (1912). *The Mosquitoes of North and Central America and the West Indies*. Carnegie Institute of Washington. I. 152 pp.
- MCGREGOR (1916). *Bulletin of Entomological Research*, VII. 81-85.
- MITCHELL (1907). *Mosquito Life*, p. 145. G. P. Putnam and Sons, New York and London, 1907.

¹ Both quotation and record are taken from Howard, Dyar and Knab (1912), Vol. I. p. 281. We have been unable to refer to this book in London. There is no reference to it in the *Zoological Record*.