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A NUTRITIONAL STUDY OF INSECTS, WITH SPECIAL REFERENCE TO MICROÖRGANISMS AND THEIR SUBSTRATA[1]

J. PERCY BAUMBERGER

Bussey Institution for Research in Applied Biology, Harvard University

CONTENTS

[1] Contribution from the Entomological Laboratory of the Bussey Institute, Harvard University.

INTRODUCTION

Throughout the whole organic world the essential food element most difficult to acquire is nitrogen, as all nitrogen must ultimately come from the atmosphere and the power of combining with this gas is limited to a few microörganisms. Upon the nitrifying bacteria, then, all higher plants and animals are dependent for their nitrogen which is handed from one organism to another, linking all together into one great interdependency which has

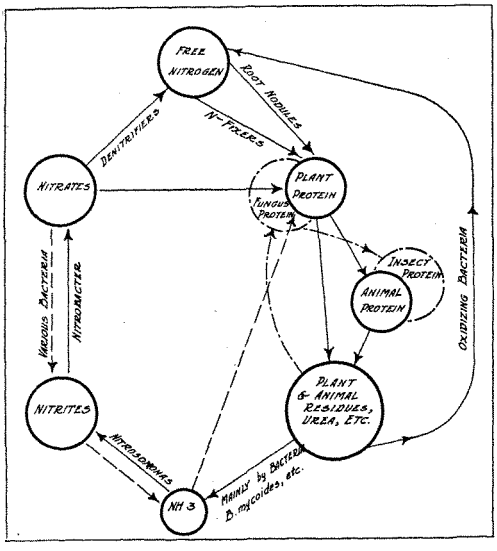


Fig. 1 The nitrogen cycle (from Bayliss). The accessory lines and circles in — - — - — are my additions based on evidence in this paper.

been called the nitrogen cycle. I insert a diagram from Bayliss which clearly illustrates this cycle. The accessory circles and the lines that connect them are additions based on my experiments.

The search of the insect for nitrogen is very complicated and has been, at times, obscure. Indeed, little definite information is at hand concerning the food requirements in general of these organisms, as the material consumed is often in large part merely the substratum for a small amount of assimilable food. This has led to many misunderstandings as to the synthetic power of

insects. Since they are largely phytophagous, insects are amply supplied with carbohydrates, but have difficulty in obtaining sufficient protein. The abundance of the former permits great activity, while the dearth of the latter limits the growth of the insect. This has led to a lengthening of the life-cycle in those species which must ingest large quantities of substrate in order to get enough nourishment to complete their growth. However, many insects that feed in decaying of fermenting vegetable matter of low protein content have an unusually short period of growth. The experiments and considerations which follow throw light on the protein supply of such insects and account for their rapid growth.

These investigations were made at the Bussey Institution for Research in Applied Biology, Harvard University, under Prof. W. M. Wheeler. Valuable advice and assistance were received from Profs. C. T. Brues, W. J. V. Osterhout, I. W. Bailey, and Dr. R. W. Glaser. I am especially indebted to Doctor Wheeler for helpful suggestions and encouragement.

EXPERIMENTS

1. Food of an insect (Drosophila) living in fermenting fruit

A. Method and initial observations. *a.* Solid media for Drosophila. While rearing Drosophila it was found necessary to determine the exact date of oviposition. As this is impossible in the ordinary culture tube of fermenting banana, a solid transparent medium was devised by myself and Dr. R. W. Glaser (1917 a).

This medium is made as follows: Mash six ripe bananas in 500 cc. of water, allow to infuse on ice overnight, strain through cheese-cloth, and add $1\frac{1}{2}$ grams powdered agar-agar to each 100 cc. of the filtrate. Heat in double boiler till agar is dissolved, filter hot through absorbent cotton into test-tubes. Plug tubes, sterilize in autoclave, and allow to cool in inclined position so as to form solid slants of the medium.

This medium is quite transparent, affords 15 to 20 sq. cm. area for oviposition and 6 to 10 cc. of substratum for the larvae. The

eggs, which are readily deposited by the female, are prominent objects on the agar.

Bacterial and fungous growths occur over the surface, but I noticed that unless these become too luxuriant before the larvae hatch, they are destroyed by the insect.

The agar method has the advantage of permitting observation of the date of egg deposition and hatch and the details of larval habits. It also furnishes a method of making nutritional studies of various synthetic media.

b. Preliminary observations on the food of Drosophila. In May, 1916, while rearing Drosophila melanogaster on banana agar, I noticed that molds and bacteria often completely covered the surface of the medium and killed the larvae. This was confined to cultures which had only ten or twenty instead of the usual fifty or a hundred larvae. The larvae congregated at the points where fungus was most abundant and caused the plants to disappear, apparently by feeding upon them.[2] An examination of the flora showed that Saccharomycetes were invariably present and often occured in pure cultures.[3]

This observation suggested an internal symbiosis between Drosophila and yeast. I found, nevertheless, that by washing the surface of the pupae with alcohol, the insect could be freed from all microörganisms. The larvae of such sterile insects were not able to mature on banana agar nor could they mature on a synthetic medium of salts and sugars with ammonium tartrate as the source of nitrogen, as had been maintained by Loeb ('15[4]), but were able to develop on either medium in the presence of yeast cells.

c. Habits of adults and larvae. The Drosophila were introduced as pupae, usually three being placed on the side of the test-tube. The adults emerge after five to eight days, the time depending on the temperature, and readily feed on the banana medium,

[2] This interpretation was first suggested to me by Mrs. J. Jackson.

[3] In 373 transfers of pupae, all descendants of adult Drosophila, taken from a stock bottle of fermenting banana, all tubes were infected with yeast cells carried on the bodies of the insects.

[4] Loeb has since corrected this view ('16). Loeb and Northrop ('16 b).

on which they leave little depressed spots where they have regurgitated and sucked up the dissolved substance. If the medium has not dried enough to have taken on a hard, leathery crust, the females oviposit after twenty-four hours and continue to do so for some days. The eggs are thrust into the agar so that the upper end with its two projecting floating structures is just level with the agar; in this position they are prominent objects under the binocular. After a period of one or more days, the minute larvae leave the eggs and move about over the surface of the medium. They are at this time usually 1.2 mm. in length. By the second day they have increased in size to 1.8 to 2 mm. in length, and begin to work in a vertical position, with the anterior end down, the full length of the body in the jelly, and the posterior end with its two projecting spiracles either in contact with the air or with a bubble of air which has been enclosed in a thin film of the medium and remains attached to the larva, thus enabling the latter to work the food material to a greater depth than its body length would permit. The head end of the larvae is merely a small pointed segment which served as a collar through which the pseudo-maxillary apparatus works. In shape the latter may be roughly compared to a plow with the shares prolonged posteriorly into two handles. Attached at the anterior end of this four-pointed median structure, is a pair of deflected falcate processes, sharp at the point and on the concave side, that work up and down constantly with a simultaneous backward and forward movement of the whole apparatus. The movements of these oral organs were observed in a drop of agar on a depression slide, and it was found that their constant movement continued without any appreciable rest periods. Occasionally the movement would stop without apparent reason for about two minutes, but there was no regularity in these periods of cessation. The larva might work for fifteen minutes without stopping or might stop several times at intervals of two or three minutes. Apparently the recovery from fatigue takes place in the interval between the movements. Progression of the larva seems to be due to a series of protrusions of the anterior end with an accompanying circular contraction, the animal being held in place by the circles of spines on each seg-

ment, while the posterior end is drawn up. In more fibrous material, the mouthparts probably aid the larva in moving about. When fully grown, it leaves the medium to pupate on the side of the test-tube or the surface of the medium itself.

d. Ecology of cultures. Drosophila is very extensively used by geneticists in breeding experiments. The insect is reared in small glass bottles or milk jars, plugged with cotton and containing fermenting banana covered with absorbent paper. Quite often these 'cultures go bad,' i.e., smell strongly of acetic acid or become putrid or covered with mold, so that the insects are destroyed and the breeding experiment terminated.

The method commonly employed in making the culture media is to boil skinned bananas, to cool the mass and to add two cakes (24 grams) of Fleishmann's bread yeast (bottom yeast) per dozen bananas. This is allowed to ferment and is used as a stock supply from which to prepare clean culture bottles. In this manner the medium is kept fairly sweet, probably due to the great development of the yeast, with an accompanying production of alcohol which retards[5] the development of molds and bacteria.

If pupae are taken from a bottle that has gone 'bad' and placed on banana agar, a number of different bacteria or molds may develop around then, prominent among which are a mucor, Rhizopus nigricans Ehrenberg, the bread mold, Aspergillus, the green herbarium mold, Penicillium glaucum, the blue mold, and the acetic acid bacillus. If pupae are taken from a good culture tube with yeast alone or yeast and the acetic acid

[5] In this connection Lafar ('10, II, 2, pp. 238–240) writes: "From the standpoint of the oecological theory of fermentation, the alcohol produced by yeast should be regarded as a weapon capable of hindering the appearance of other fungoid competitors in saccharine nutrient media. However, when accumulated in the medium during the progress of fermentation, it also restricts the further development and action of its producer. In this case, as with yeast poisons in general, the first result is the cessation of cell reproduction, a larger quantity of alcohol being necessary to arrest fermentation and a still further quantity to kill the cells." Reproduction of yeast cells ceases at a 6 per cent and fermentation at a 5 to 24 per cent concentration of alcohol. It should be also remembered that most bacterial or fungus cultures have a tendency to become pure, probably owing to the production of some definite antagonistic substance, or to better adaptation to the medium by the successful form (Hiss and Zinsser, '10).

bacillus, the Drosophila larvae grow rapidly, the fungous growth soon diminishes and is visible at only a few points on the surface. If the flora contained molds, the whole surface of the medium is soon covered and the Drosophila eggs are killed, or more often hatched and the young larvae die. If the mold does not completely cover the surface, many larvae survive, and upon increasing in size, are able to destroy the mass of mold hyphae and form a fairly clean surface. The larvae are able to do this only when they are in large numbers and have reached a size of 3.5 to 4.5 mm. before being covered by the molds. It would seem, therefore, that the destructive action of the molds is mechanical rather than toxic. It was also observed that molds seldom gain a foothold on media in which large numbers of larvae are feeding. This observation explains why 'strong' cultures of Drosophila (as usually reared on fermenting banana) remain 'sweet' and seldom go bad. Banana-agar culture tubes in which the Drosophila pupae have been placed on the glass, rather than on the medium itself, often remain sterile till the adults emerge. The latter spread the spores over the surface of the agar at the same time that they deposit their eggs. Thus the molds and bacteria have little time to grow before the larvae are at work.

The development of molds and bacteria is not apparent in the presence of large numbers of larvae and a strong culture of yeast in the proper nutrient medium.

e. Media for genetical work. In selecting the best medium in which to rear Drosophila the most important considerations are abundant food for the yeast cells and a moist jellylike consistency of substratum to which the larvae are adapted. Transparency and solidity of media will add to the convenience of the investigator.

I have obtained the best results by using Saccharomyces ellipsoideus, in the stock bottle of banana, as the fragrant odors of fermentation produced by this yeast stimulate oviposition by the fly. The two following media have proved most satisfactory: 1. Fermented banana agar. Ferment one dozen mashed bananas for 48 hours, strain through cheese-cloth, add agar, sterilize and slant. 2. Pasteur's culture fluid agar.

10 grams ashes of yeast
10 grams ammonium tartrate
100 grams rock candy
1000 grams water

Add agar, sterilize in Arnold sterilizer, slant.
Into sterile tubes of these media the introduced adults or pupae carry living yeast cells which are distributed through the medium by the activity of the larvae.

f. Are living yeasts present in the egg or pupa? In the following experiments undertaken to show that microöganisms are not transmitted through the egg of Drosophila, the first precaution was to free the insect from external microörganisms. Usually eggs are used for this purpose, but the small size of Drosophila eggs makes this a difficult procedure. As it is well known that the lining of the digestive tract of larvae is thrown off upon pupation, pupae were selected for sterilization.

The pupae from a culture strong in yeasts were submerged in 85 per cent alcohol for ten minutes and then introduced aseptically into sterile slant culture tubes of agar-agar and fermented banana filtrate. If no yeast developed around the pupae which were placed on the food, the tube remained sterile after the emergence of adults, oviposition, and hatching of larvae. The sterility of the tube was later tested by introducing a few loopfuls of the medium into a sterile tube of similar food. It had previously been determined that yeast developed readily on fermented banana agar.

2. Larvae which had been feeding on media containing living yeast cells were submerged and washed in 85 per cent alcohol and then introduced into sterile culture tubes. In all cases yeast developed on the new media. Cultures from the digestive tracts of the larvae gave similar results. Apparently, many cells escape digestion in the stomach, as is the case with seeds or insect eggs in birds.

3. Eggs were sterilized by soaking in 85 per cent alcohol for ten minutes. The larvae which hatched were always sterile.

From the foregoing experiments we may conclude that living microörganisms are not present in the eggs or pupae of Drosophila.

However, a loose symbiosis exists between yeast and the insect. As mentioned above, surface fungous growths disappear in the presence of larvae which often seemed to be more numerous at this point. From these observations I inferred that the larvae fed upon the microörganisms present.

g. Sterilization of pupae. The sterilization was accomplished by the use of ethyl alcohol. As a precaution the operator's hands were washed in alcohol, and a lighted burner, clean forceps and platinum loop as well as sterile culture tubes were ready on a

TABLE 1

CULTURE NO.	NO. PUPAE	ALCOHOL TREATMENT	NO. ADULTS EMERGED	LARVAL PERIOD	CONTAMINATION
A 8	2	50% 10 seconds	2	None	Yeast cells
A 9	2	50% 5 seconds	2	None	Yeast cells
A 10	3	50% 20 seconds	3	12 days p[1]	Yeast cells
A 11	3	50% 2 seconds	3	11 days p	Yeast cells
A 12	3	50% 2 seconds	3	14 days p	Medium brown, yeast coccus, rod
A 13	2	50% 2 seconds	1	None	Yeast cells (?)
A 17	3	85% 2 minutes	3	26p	Yeast cells
A 18	8	85% 5 minutes	6	28d[1]	
A 19	5	85% 6 minutes	5	None	
A 20	6	85% 7 minutes	6	26p	Yeast cells
A 24	5	85% 10 minutes	4	25d	
A 25	7	85% 10 minutes	6	44d	

[1] d indicates larval death
p indicates pupation

table also washed with alcohol. Pupae were taken from a tube having a strong growth of yeast, but uncontaminated by molds[6] and placed in a sterile watch-glass. Alcohol was then poured in till the pupae were submerged. All floating pupae and all larvae were removed. The results of this treatment for different periods of time are shown in table 1.

The pupae are able to withstand a treatment of 25 minutes in 85 per cent alcohol if applied when they are about two days old.

[6] The frequency with which pure yeast growths occur in Drosophila cultures has already been mentioned on page 4.

Treatments of five minutes seldom kill the pupae, and in 90 per cent of the cases render them sterile. The sterilizing effect was not entirely understood till pupae were used which came from a Drosophila stock bottle of fermenting banana contaminated by molds and bacteria. These pupae when washed with 85 per cent alcohol saturated with $HgCl_2$ were sterilized in less than 50 per cent of the cases as shown in table 2.

This indicates that the sterilization involves two stages, 1) destruction of molds and bacteria by feeding of the larvae and a good strong yeast growth and, 2) killing of yeast by alcohol.

The toxicity of alcohol for yeast cells is shown to be high by the following experiments:

Three grams of yeast were separated in 25 cc. of sterile water and two drops of this fluid were added to each of ten watch-glasses filled with 85 per cent alcohol and to ten sterile banana-agar tubes.

TABLE 2

NO. CULTURES	NO. PUPAE	TREATMENT	NO. ADULTS EMERGED	NO. TUBES CONTAMINATED	NO. PUPAE CONTAMINATED
15	150	85 per cent alcohol sat. with $HgCl_2$	15	8	83±

After 1, 5, 10, 15, and 20 minutes, respectively, two sterile banana agar tubes were inoculated with two drops of yeast from the watch-glasses of alcohol. The tubes were kept under observation for 21 days. The results in table 3 show that a five-minute exposure to 85 per cent alcohol is fatal to yeast cells.[7]

h. Test of sterility. The sterility of a culture tube could usually be judged by the fact that no growth occurred, 1) around the pupae which were placed on the medium, 2) at spots where adults regurgitated on the medium, 3) at adult fecal spots, 4) upon oviposition, 5) upon emergence of larvae, 6) upon pupation of larvae. If no growth occurred in the first case, i.e., around the pupae, the medium showed no sign of contamination throughout the life-cycle. Bacterial growths visible around the pupae might disappear during the life of the larvae, but usually reappeared when

[7] Paine ('11) showed that yeast cells are highly permeable to alcohol which readily and permanently plasmolyzes them.

the larvae pupated. Apart from visible growths, the sterility of the tube was tested by introducing loops full of the medium on which larvae were working or had pupated into a sterile tube of one of the following media; potato agar, banana agar, Pasteur's agar, nutrient gelatine, nutrient bouillon and yeast agar. One or all of these media were used to test the environment of the larvae for the presence of microörganisms (fig. 18). Usually crushed adults, pupae, or larvae were also introduced into the test culture tube. The method of inoculation was by stab or streak; in the former case semianaërobes could develop. Banana agar was used most often, as it more nearly resembles the natural environment of the fly and its associated organisms and also can support vigorous growths of a large flora.[8]

TABLE 3

NUMBER OF INOCULATIONS	TIME OF EXPOSURE TO 85 PER CENT ALCOHOL	NUMBER OF CONTAMINATIONS
	min.	
10	0	10
2	1	2
2	5	0
2	10	0
2	15	0
2	20	0

Smears of the media were examined after staining in the usual manner with eosin or Loeffler's methylene blue. This examination was made with a 1.6 mm. Zeiss objective. Fresh smears were examined, before staining, with dark and light field illumination.

B. Food of Drosophila. *a.* Growth of sterile larvae on sterile fruit. From the foregoing experiments it is clear that yeast is always present in the habitat of Drosophila larvae and is usually imported into sterile media on the body of the adult or pupa. Pupae and eggs do not contain living yeasts and any yeasts on the external body surface can be killed by alcohol.

[8] For example, Cocobacillus acridiorum, Bacillus prodigiosus, B. coli, B. aceti, Streptococcus dispar, Saccharomycetes cerevisiae, S. ellipsoideus, S. anomalus, Penicilium glaucum, Rhizopus nigricans, Aspergillus, Fusarium, Gliocladium, etc.

If sterile pupae are placed on a sterile medium of banana agar and protected from contamination, the adults emerge and oviposit, but the larvae that hatch develop very slowly and finally die before pupating. The great difference in rate of growth between sterile and non-sterile larvae on the same food is shown in figure 2. In cultures A 10, 11, 12, amd 17 living yeast cells were present and the larvae grew at a normal rate, reaching the full length of 8 mm. in eleven to twenty-six days when pupation took place. In cultures[9] A 18 and 25, on the other hand, the sterile larvae reached a size of only 3 mm. after twenty-eight to forty-

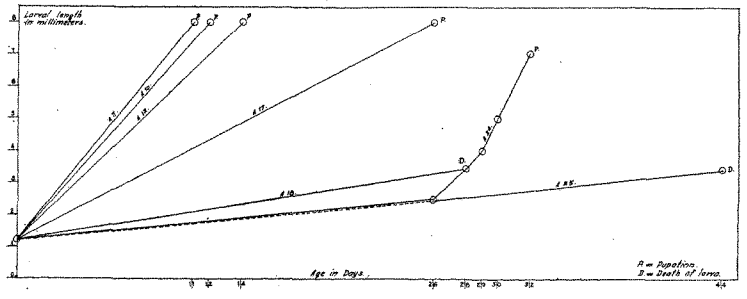


Fig. 2 Larval growth on banana agar. A 10, 11, 12, 17, growth in cultures infected with living yeasts; A 18, 24, 25, slow growth of larvae in sterile cultures; A 24, infected with living yeast on twenty-sixth day, causing an increase in growth.

four days, when they died. In culture A 24 the sterile larvae reached a length of 2 mm. in twenty-six days when the medium

[9] The size of the larvae on different media was determined by placing the tubes and a millimeter scale on the stage of a binocular microscope and measuring the length of five to ten of the larger specimens while 'crawling' at full length. The larger specimens were selected for measurement because, although female adults were allowed to oviposit for only one day, the eggs showed considerable variability from one to three days in their date of hatching, depending on the readiness with which the female oviposited on the medium.

The cultures were kept in a steam-heated room in which the maximum temperature for the entire period of experimentation varied between 96° and 71°F. and the minimum between 73° and 56°F. As compared experiments were run parallel in time, the error due to temperature differences should not be great.

It should be kept in mind that each point on a curve of growth is the average of the whole culture of larvae, i.e., usually twenty or more individuals, thus a single curve has considerable weight.

was inoculated with living yeast. This caused a rapid increase in size and ended in pupation six days later.

The acceleration which takes place on infecting a sterile medium with living yeasts indicates that the alcoholic treatment in sterilizing the pupae does not cause the decrease in the rate of growth of the sterile larvae. Other cases of acceleration which occurred due to accidental contamination of a sterile medium quite often bore out this conclusion.

Therefore, it is certain that sterile larvae grow more slowly than non-sterile larvae on sterile food, and that the rate of growth can be increased by infecting the medium with the living yeast.

b. Is fruit the food for Drosophila larvae or merely the substratum for yeast cells? As sterile larvae grow so slowly and do not pupate in sterile fruit, but develop normally if it is infected with living yeasts, the question arises as to the true position of the fruit in the ecology the insect. By using a medium containing the inorganic salts and the sugars and ammonium tartrate necessary for yeast growth, the starch, oils, fats, proteins, and other substances of the fruit were eliminated from the experiment.

The composition of the medium was as follows:

Agar-agar	4.0 grams	K_2HPO_4	0.165 grams
Grape-sugar	16.5 grams	$MgSO_4$	0.165 grams
Cane-sugar	16.5 grams	H_2O	200 cc.
Ammonium tartrate	3.3 grams		

Sterile larvae lived only five days on this sterile medium and showed no increase in size; but if the medium was infected with living yeasts, the larvae grew at a normal rate, reaching their maximum size in ten days, and pupated normally. The adults which emerged from these pupae were sexually fertile and of large size. Thus, in the presence of living yeast, Drosophila larvae grow normally in a synthetic nutrient medium for yeast with ammonium tartrate as the only supply of nitrogen. Therefore the simplest nutrient medium for yeast if infected with living yeasts is equavalent to fermenting fruit in the ecology of Drosophila larvae.

The nutrient medium for yeast in itself is not an adequate substitute for sterile fruit, as Drosophila larvae live longer on the latter, e.g.,

Medium	*Increase in Length*	*Longevity*
Sterile banana agar	1.8 mm.	26 to 44 days
Sterile yeast nutrient medium	0 mm.	5 days

Therefore sterile fruit has greater food value for sterile larvae then the simplest 'nutrient medium for yeast.' Fruit is mainly the nutrient substratum for yeast cells, but has some food value for Drosophila larvae.

c. Are products of fermentation essential food requirements of Drosophila larvae? In the preceding experiments living yeast cells had an opportunity to develop and form products of fermentation in the media. As these products may have food value for the larvae, the essential difference between a septic and a sterile food might be the absence of these substances. If this were the case, the larvae would be dependent on yeast not as a food, but as a chemical agent.

By boiling yeast before adding it to yeast nutrient agar, the formation of fermentation by-products was prevented. Fleishmann's bread yeast was used for this purpose and 6 grams were added to every 100 cc. of yeast nutrient agar. On this medium sterile larvae grew at a normal rate, reaching their full size in ten days and pupating normally. This proves that Drosophila larvae grow normally on dead yeast in the absence of any by-products of fermentation.

d. Is yeast a complete food for Drosophila larvae? In the media used thus far various substances besides yeast were present. To eliminate these and determine whether or not yeast alone is a complete food for Drosophila larvae, media were made up of Fleishmann's compressed bread yeast, water, and agar-agar.[10] It was found that sterile larvae on a medium of 6 grams yeast per 100 cc. water grew as rapidly as non-sterile larvae and many times faster than sterile larvae on banana. In figure 3 cultures W 3, 4, and 5 show that larvae on dead yeast grew to maximum

[10] Sterile larvae live a maximum of five days on sterile 1½ per cent agar and water medium, showing no increase in size.

size in four or five days, while larvae on sterile banana (A) did not reach their maximum size in twenty-eight days.

The minimum requirement of yeast was found by the use of media consisting of 1, 2, 3, 4, 6, 9, 12, and 24 grams of yeast, respectively, separated in 100 cc. of water and thickened with powdered agar-agar. On sterile 1 per cent yeast the larvae grew very slowly for twenty days, dying at a length of 4 mm. without pupating. On sterile 2 per cent yeast larvae pupated when 5.5 mm. in length, reaching this size on the 10th day. On 3, 4, 6, 9, and 12 per cent yeast media the results were much alike, the larvae

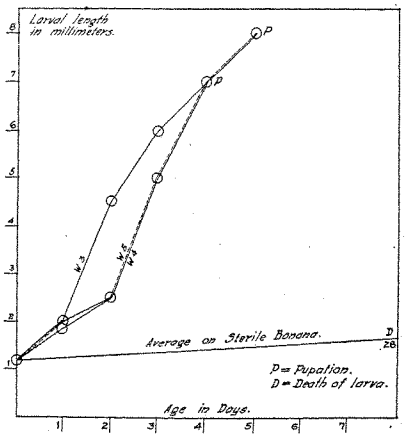


Fig. 3 Larval growth on dead yeast. W 3, 4, 5, show rapid growth on dead yeast; A, shows slow growth on sterile banana.

reaching a size of 7.5 to 8 mm. in length on the third, fourth, or fifth day and pupating before the eighth day. On 24 per cent yeast the larvae often reached a length of 6.5 mm. on the first or second and pupated before the sixth day.

Records of the growth of cultures of larvae, on yeast media of different strengths, follow in table 4 and the mean larval periods are included in table 5. In figure 4, curve 1 shows the rapid growth on 24 per cent yeast; curves 2 and 3, the maximum and minimum rates of growth on 3 to 12 per cent yeast, and curves 7, and 9, the slow growth on 2 and 1 per cent yeast.

TABLE 4

Age of larvae in days

MEDIA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	20	26	27	28
	Length in millimeters																			
Hot aqueous unfermented banana sol.....1		2	3				4				4.5			5		5.5	5.5	5.5	6	P
Campestris.....1				2.5	2.5		5	6				P								
1 per cent yeast.....2		2			2	2.5	3	3	3	3	3		3				4			
2 per cent yeast.....0			2	2.5	2.5	2.5	3.5	5.5	5.5	5.5		P								
3 per cent yeast.....1	2	3		8	8			P												
3 per cent yeast.....2		5.5	6	7		P														
3 per cent yeast.....3		3	5	8			P													
4 per cent yeast.....2	2	3.5	6.5	7	7.5		P													
4 per cent yeast..... 3	2	3	6	7	7			P												
6 per cent yeast.....1	2.5	4		7.5	P															
6 per cent yeast.....2		5	6.5	8	8	P														
6 per cent yeast.....4	2	3.5	6	7	8		P													
12 per cent yeast.....2		6	8			P														
12 per cent yeast..... 3	2	4.5	6	7	8		P													
12 per cent yeast.....4	1.8	3	5	7	7		P													
12 per cent yeast.....5	2	2	5	7	8			P												
24 per cent yeast.....1	6.5	7	7.5			P														

TABLE 5

MEDIA	NO. PUPAE	MEAN LARVAL PERIOD	STANDARD DEVIATION	COEFFICIENT OF VARIABILITY	NO. ADULTS	MEAN PUPAL PERIOD	STANDARD DEVIATION	COEFFICIENT OF VARIABILITY
Hot aqueous sol. unfermented banana	8	20.			3	5.		
	4	28.75	1.29	4.5	9	5.5		
Banana mash.....1	1	11.0			0			
Banana mash.....4	2	13.0						
Agaricus campestris.....1	14	12.43	1.89	15.2	14	3.85	0.6221	16.1
1 per cent yeast.....2	0	20.						
2 per cent yeast.....1	10	11.4	1.42	12.4				
3 per cent yeast.....1	29	7.14	0.27	3.78	29	4.75	0.2318	4.8
3 per cent yeast.....2	10	6.00	0.14	2.03				
3 per cent yeast.....3	50	6.32	1.93	30.5	40	4.33	0.0409	0.9
4 per cent yeast.....1	28	13.05[1]	1.55	11.1				
4 per cent yeast.....2	36	6.47	1.70	26.0	33	6.63	11.66	25.0
4 per cent yeast.....3	40	7.20	1.208	14.00	30	5.58	2.89	43.59
6 per cent yeast.....1	17	5.00	0.0	0.0	20	4.0	0.0	0.0
6 per cent yeast.....2	21	6.0	0.308	5.1	20	4.4	0.154	3.5
6 per cent yeast.....4	61	6.46	0.157	2.4	60	4.37	0.2454	5.6
12 per cent yeast.....2	40	5.8	0.5	8.8	40	5.45	1.07	19.6
12 per cent yeast.....3	51	7.0	0.713	10.2	51	4.46	0.6361	14.2
12 per cent yeast.....4	17	6.3	0.525	8.3	17	4.17	0.41	9.8
12 per cent yeast.....5	50	7.8	1.095	14.0	25	3.68	1.2	32.6
24 per cent yeast.....1	60	5.21	1.61	30.9	60	5.44	0.527	9.6
24 per cent yeast.....2	12	4.83	0.188	3.8	12	3.93	2.02	56.0
3 per cent yeast.....average	89	6.55			69	4.50		
4 per cent yeast.....average	76	6.85			66	5.85		
6 per cent yeast.....average	79	6.11			100	4.30		
12 per cent yeast.....average	158	6.87			133	4.07		
24 per cent yeast.....average	72	5.14			72	5.18		

[1] F_1 from adults reared on Agaricus campestris.

These experiments show that dead yeast is an adequate food for Drosophila larvae when in a concentration of 2 per cent or more.

e. Can Drosophila larvae complete their growth on any vegetable food other than yeast? Bacteria and fungi other than yeasts appear to have some food value for Drosophila larvae, as in microscopic examinations of the digestive tract bacteria often form the bulk of the contents. The following experiments show

that these microörganisms are not as valuable to the insect as yeast cells. A few larvae were reared on vinegar-plant agar, pupating on the sixth day. On manure agar growth was slower and pupation took place on the fifteenth day. On lactic acid bacillus and on Rhizopus nigricans agar no growth took place, but the larvae died in three to five days. On plain agar infected with a semianaërobic bacterium a few larvae pupated after twenty-six days. Therefore yeasts are a more complete food for Drosophila larvae then other bacteria or fungi.

I have already shown that fruit (banana) is of some food value for Drosophila larvae, as it will keep the insects alive for periods

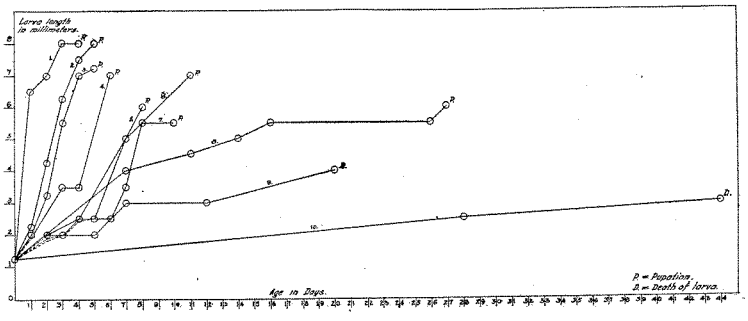


Fig. 4 Larval growth on various media. 1, 24 per cent yeast; 2, maximum 3 to 12 per cent yeast; 3, minimum 3 to 12 per cent yeast; 4, vinegar plant; 5, mushroom; 6, yeast nucleoprotein, sugars, and salts; 7, 2 per cent yeast; 8, hot aqueous extract of banana; 9, 1 per cent yeast; 10, cold aqueous extract of banana.

of twenty-eight to forty-four days and permit them to increase in size to a limited extent.

The activity of the larvae and analysis of the banana indicate that the insect is abundantly supplied with carbohydrates (20 per cent sugar in ripe fruit). The protein content, on the other hand, is relatively low (1 per cent) and is probably deficient.

The long life of the larvae on sterile banana with the accompanying slow increase in size, indicates that all the food elements required for maintenance and repair of tissues are present, but the protein content is either too small or lacking or deficient in some amino-acid necessary for growth. There is also the possibility

that some vitamine may be absent or may have been destroyed by the high temperatures of the autoclave.

Some light is thrown on these questions by a comparison of the rates of growth of larvae on banana media which have been more highly concentrated by partial desiccation or by extraction with hot water. The growth of insects on these media is shown in table 6 and figure 5.

On sterile food consisting of mashed whole bananas, especially when they have dried out slightly and are thus concentrated, an occasional small pupa is formed. On a hot aqueous extract of banana a larger number is formed from which small adults emerge.

TABLE 6

MEDIA	LARVAL PERIOD (20 OR MORE LARVAE IN EACH CASE)	NO. PUPAE	NO. ADULTS	CONTAMINATION
Banana mash 1	11 days	1	0	−
Banana mash 2	7 days	16	16	+
Banana mash 3	22 days	1	1	−
Banana mash (slightly shrunken) 4	13 days	2	2	−
Cold aqueous extract of fermented banana	28 days (Average)	0	0	−
	12 days (Average)	73	70	+
Cold aqueous extract of unfermented banana	25 days (Average)	0	0	−
	13 days (Average)	90	85	+
Hot aqueous extract of unfermented banana	20 days	8	8	−
	29 days	9	9	−
	9 days (Average)	68	66	+

These are potentially fertile, for when fed with yeast, the females deposit fertile eggs from which normal larvae emerge. This will be discussed below at greater length. On cold aqueous extracts of unfermented banana, no larvae pupate.

These results show that concentrated banana permits complete and more rapid growth of larvae. Therefore, the fruit is not entirely lacking in any amino-acid necessary for growth nor is any vitamine absent or destroyed by sterilization. It appears, moreover, that concentrated banana forms a complete food for Drosophila larvae, i.e., the protein deficiency of fruit is quantitative rather than qualitative.

As Agaricus campestris has a protein content of 3.5 per cent as compared with the 1 per cent protein content of banana, this fungus was used as a food for larvae. Drosophila females oviposited readily on a medium of powdered Agaricus campestris, water, and agar. The sterile larvae grew rapidly to a size of 6 mm. (the maximum size on yeast is 8 mm.) and pupated after an average of 12.43 days. The curves of growth are shown in figure 6 and a typical record in tables 4 and 5. The adults that emerged from these pupae were sexually fertile, but were quite small. A generation of larvae reared from some of these adults grew more slowly than normal on 4 per

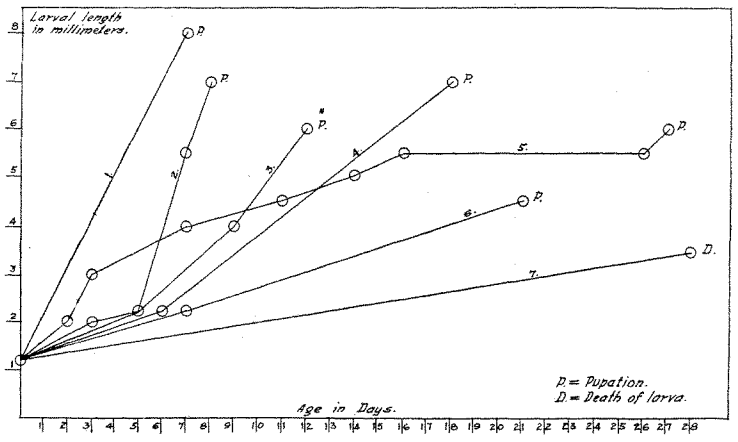


Fig. 5 Larval growth on banana. 1, mashed whole banana infected with living yeast; 2, 3, 4, sterile mashed whole banana; 5, 6, hot aqueous extract of banana; 7, cold aqueous extract of banana.

cent yeast, requiring thirteen days instead of seven days to reach maturity (fig. 6, curve 2, and table 5). Sexually fertile adults can be reared from larvae fed on mushrooms, but such adults are undersized. Agaricus campestris meets more nearly the food requirement of larvae than banana; this may be due to the higher percentage protein content of the mushroom or to a relatively higher content of certain necessary amino-acids.

f. Is yeast a more adequate food than fruit because of its high protein content? The slow growth and small size of larvae and their failure to pupate when reared on sterile fruit are typical

symptoms of protein deficiency. This deficiency is quantitative rather than qualitative, because more normal growth of the larvae is permitted when the fruit is concentrated.

As these symptoms of malnutrition are not shown in larvae reared on yeast, we would expect to find a high protein content of adequate components in this food. This assumption is correct, for Atwater and Bryant ('02) have found by analysis that the percentage protein content of yeast is 11 per cent. This is higher than the percentage occurring in any fruit or in Agaricus campestris, and Meisenheimer ('05) has shown that most monoamino-acids occur in yeast protein.

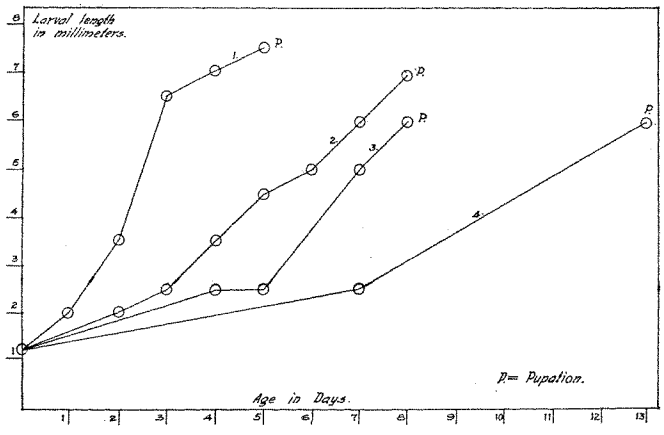


Fig. 6 Larval growth on mushroom. 3, 4, mushroom; 1, normal growth on 4 per cent yeast; 2, growth of larvae of mushroom-fed adults on 4 per cent yeast.

Is the rapid growth of larvae on yeast due to this high protein content or to other substances present in the yeast cell, such as glycogen, fat, gums, hemicelluloses, etc.? By extracting the yeast nucleoprotein and making media with known sugars and salts, this problem was solved. The method employed to extract the yeast nucleoprotein and to make the media is described in a foot-note below.[11]

[11] The pure yeast nucleoprotein was obtained in the usual manner (Hawk, '16), as follows: the cells in 4 pounds of baker's yeast (Fleishmann's bottom bread

The growth of larvae on yeast nucleoprotein media is shown in figure 7 and table 7. Table 7 shows that larvae do not grow on nucleoprotein in the absence of Pasteur's solution, but, if these salts and sugars are added, do grow rapidly and form large numbers of normal pupae from which normal highly fertile adults emerge.

yeast) were killed by ether and broken open by grinding in a mortar with a quantity of pure white diatomaceous earth, adding enough water to keep the mass in a sticky, smooth condition. The grinding was continued till examination with a 1.6-mm. objective showed that many of the yeast cells had been cut into irregular rectangles. The yeast was then poured with the addition of 0.4 per cent NaOH solution into a large bottle and 8000 cc. of the alkali were added. About 40 cc. of chloroform were then mixed with the solution to prevent the development of bacteria. The contents of the bottle were thoroughly agitated several times a day. After forty-eight hours the supernatant fluid was poured off, leaving a great part of the yeast and diatomaceous earth in the bottle. This fluid was centrifuged in a large-sized electric centrifuge with a capacity of four 250 cc. bottles. Each liter was run for twenty minutes (fifteen minutes at maximum speed) and the supernatant fluid carefully poured off. This fluid was examined for yeast cells with a 1.6-mm objective and showed an entire absence of them. After all the yeast cells had been removed in this manner, the liquid had a clear opalescent color and proved to be rich in nucleoprotein. This was precipitated in great white floccules by adding 10 per cent HCl in drops. The precipitate dissolved in the alkaline or neutral solution, but remained in the slightly acid solution in which the largest amount was formed. The precipitate was separated pure from the solution by centrifuging and washing with acid alcohol and neutral alcohol in which it was insoluble. The white precipitate was then dried over H_2SO_4, forming a white powder. The remaining fluid was neutralized with N/10 NaOH and dialyzed for five days in running water, keeping the surface covered with toluol. No precipitate was formed. The neutral, salt-free solution was then heated to boiling and acidified with a drop of HCl or acetic aid and also acidified and then boiled. No marked precipitate was formed. Half saturation, complete saturation with $(NH_2)SO_4$ when hot or cold and saturation with NaCl and with picric acid failed to bring down any precipitate. A heavy precipitate which appeared to be a peptone decomposition product of the nucleoprotein was produced upon the addition of phosphomolybdic acid.

The nucleoprotein was ground in a mortar and then made into media as follows: 1) Nucleoprotein moistened with tap-water was placed in test-tubes and sterilized in the autoclave; 2) nucleoprotein moistened with Pasteur's nutrient solution (grape-sugar, cane-sugar, ammonium tartrate, $MgSO_4$, K_2HPO_4, H_2O) in test-tubes and sterilized; 3) nucleoprotein and 1.5 per cent agar-agar tap-water solution autoclaved and mixed aseptically in sterile test-tubes, and, 4) nucleoprotein. Pasteur's nutrient solution and 1.5 per cent agar solution autoclaved and mixed aseptically in sterile test-tubes. If mixture is made before autoclaving the protein adsorbs (?) the agar and on cooling jellation does not take place.

Adults placed on media from which sugars were absent died after one to four days, whereas those placed on media containing Pasteur's solution lived for a much longer time. The larvae on the nucleoprotein alone live for several days, but do not increase in size and are not very active. It may be that a sweet taste is necessary to stimulate them to take food or it may be that carbohydrates are necessary to furnish energy "fuel." The larvae on nucleoprotein and carbohydrates grow slowly at first, but quite rapidly after reaching a length of 3 to 4 mm. This may be due to the rather large size of the nucleoprotein crystals or to the depth

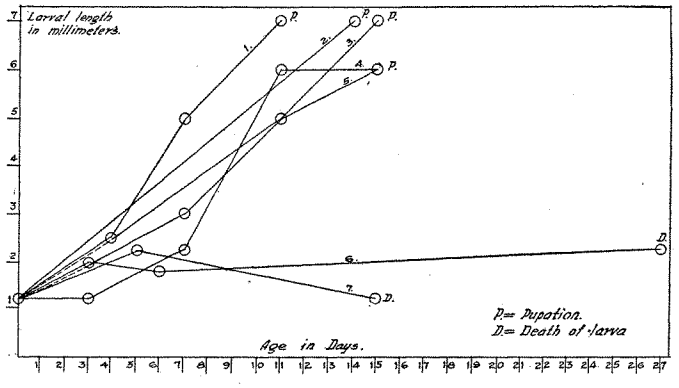


Fig. 7 Larval growth on yeast nucleoprotein. 1, 2, 3, 4, 5, yeast nucleoprotein, sugars, and salts; 6, 7, yeast nucleoprotein and tap-water.

to which they sink in the agar. In figure 7 are shown the curves of growth on these media. Curves 1 to 5 show the rapid growth on yeast nucleoprotein, sugars, and salts and curves 6 and 7 show the slow growth or diminution on nucleoprotein alone. One curve of growth on yeast nucleoprotein and sugar, etc., given (fig. 4, curve 6) in the same figure with curves for yeast media, shows that larvae grow more rapidly on yeast nucleoprotein and sugar than on 2 per cent but less rapidly than on 3 per cent yeast. It must be remembered that mechanical questions of ingestion and the question of taste or olfactory preference may largely affect the amount of material eaten and therefore the rate of growth.

TABLE 7

MEDIA	NO. DAYS ADULT LIVED ON MEDIUM	LARVAL PERIOD	NO. PUPAE	NO. ADULTS	CONTAMINATION
Nucleoprotein + tap water No. 1.....	2	6	3	3	+
Nucleoprotein + tap water No. 2.....	2	5	4	4	+
Nucleoprotein + tap water No. 3.....		12d	0	0	—
Nucleoprotein + tap water No. 4.....		10d	0	0	—
Nucleoprotein + Pasteur's sol. No. 1.		10	1*	0	—
Nucleoprotein + Pasteur's sol. No. 2.....		5	0*	0	—
Nucleoprotein + Pasteur's sol. + agar No. 1.	5+	22	3+	1+	—
Nucleoprotein + Pasteur's sol. + agar No. 2.	6+	16	80+	80+	—
Nucleoprotein + Pasteur's sol. + agar No. 3.		21	10+	?	—
Nucleoprotein + Pasteur's sol. + agar No. 4.	7	12	30	30	—
Nucleoprotein + Pasteur's sol. + agar No. 5.		14	3	0	—
Nucleoprotein + tap water + agar No. 1.....		5d	0	0	—
Nucleoprotein + tap water + agar No. 2.....		21d	0	0	—
Nucleoprotein + tap water + agar No. 3.....	3	16d	0	0	—
Nucleoprotein + tap water + agar No. 4.....		5d	0	0	—
Nucleoprotein + tap water + agar No. 5.....	1	5d	0	0	—
Nucleoprotein + tap water + agar No. 6.....	2.5	5d	0	0	—
Nucleoprotein + tap water + agar No. 7.....	4	0x	0	0	—

x No eggs laid.
d indicates death.
* Media dried.

Hence the objection that there is no exact mathematical correlation between the rates of growth and the protein concentration is not irrefutable evidence against the view that primarily such a relationship exists. For example, some farm animals will lose weight on a soy-bean diet of high protein value, but of a taste they do not like.

Since Drosophila grows normally, pupates in large numbers, and develops into fertile adults of good size, it appears that a medium of yeast nucleoprotein, sugars, and inorganic salts is a complete food for this insect. It has already been proved by experiment (p. 13) that larvae do not grow on sugars and inorganic salts alone, so that the nucleoprotein is the substance which, if added, makes the medium equivalent to yeast cells as food for

the insect. As the sugars and inorganic salts are abundantly present in fruit[12] and the addition of nucleoprotein of yeast is sufficient to make the synthetic medium a complete diet for Drosophila, it can be said that yeast is a more adequate food than fruit because of its high protein content.

g. Conclusions. 1. Insects can be conveniently reared in a solid agar medium.

2. Larvae prevent the development of molds on the medium, but are always associated with living yeasts.

3. For genetical work fermented banana agar or Pasteur's culture fluid agar is most convenient.

4. Living yeasts are not present in the egg or pupa.

5. The exterior of pupae can be sterilized by washing in 85 percent alcohol for twenty minutes. Yeast cells are more readily killed by this treatment than molds.

6. Banana agar is a good culture medium for fungi.

7. Sterile larvae grow more slowly than non-sterile larvae on sterile fruit; the rate of growth can be increased by infecting the medium with living yeasts.

8. The alcoholic treatment in sterilizing pupae is not the cause of the slow growth of larvae on sterile food.

9. The simplest nutrient medium for yeast, if infected with living yeast, is equivalent to fermenting fruit in the ecology of larvae.

10. Sterile fruit has greater food value for larvae than "sterile nutrient medium for yeast."

[12] Atwater and Bryant ('06) give the following analysis of the edible portion of banana:

	Water per cent	*Protein per cent*	*Fat per cent*	*Total carbohydrate per cent*	*Ash per cent*	*Fuel value per lb.*
Minimum	66.3	1.0	0.0	16.3	0.5	330
Maximum	81.6	1.6	1.4	29.8	1.1	640
Average	75.3	1.3	0.6	22.0	0.8	460

Prescott ('17) gives this analysis of banana ash:

	per cent		*per cent*		*per cent*
Silica	2.19	Phosphoric acid	7.68	Potash	43.55
Lime	1.82	Magnesia	6.45	Sulphur trioxide	3.26
Iron oxide	0.18	Soda	15.11	Chlorine	7.23

11. Fruit is mainly the nutrient substratum for yeast cells, but has some food value for larvae.

12. By-products of fermentation are not necessary for larvae.

13. Dead yeast is an adequate food for larvae when in a concentration of 2 per cent or more.

14. Yeast is a more complete food for larvae than other fungi.

15. Concentration of banana by hot-water extraction or drying makes it an adequate food for larvae.

16. The protein deficiency of fruit is quantitative rather than qualitative.

17. Agaricus campestris meets more nearly the food requirements of larvae than banana.

18. Yeast nucleoprotein, sugars, and salts are an adequate food for larvae.

19. Yeast is a more adequate food than fruit because of its higher protein content.

C. Discussion. a. Effect of food on larval, pupal and adult life. On page 15 and in table 5 and figure 4 it has already been shown that the concentration of yeast affects the length of the larval period. This effect can be seen more clearly if we plot the larval period on the axis of ordinates (vertical), of a graph, and the number of grams of yeast per 100 cc. of water, in the media, on the axis of abscissae (horizontal). A curve drawn through the points established represents the effect of the concentration of yeast upon larval life. This curve is drawn in figure 8. It changes its direction very suddenly at a point between 2 and 3 per cent of yeast, going up from a larval life of 6.55 days on 3 per cent to a period of 11.40 days on 2 per cent yeast. If we continue the curve in the same direction we approximate a period of twenty days representing the larval life (which ends in death) on 1 per cent yeast medium. This great change in the direction of the curve indicates that there is a definite concentration (2 per cent) of yeast necessary for the completion of the larval period. Between yeast concentrations 2 per cent and 3 per cent there is a difference in the larval period of 4.95 days, whereas between concentrations 3 per cent and 12 per cent there

is only a variation of 0.76 day and between 3 per cent and 24 per cent—a difference of 1.11 days. It appears that the normal condition for larval growth is in a medium of yeast concentration between 6 to 24 grams per 100 cc. This is shown in the size of pupae in table 8.

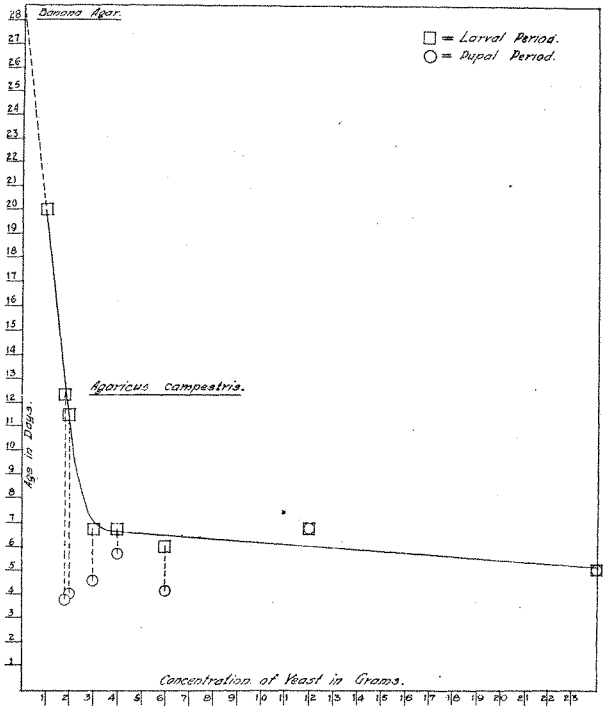


Fig. 8. The effect of the concentration of yeast on the length of the larval and pupal periods.

A concentration of 1 per cent of yeast appears to be sufficient to furnish the energy, repair, and some growth requirements of the larvae for a considerable period without furnishing quite enough food to allow the necessary growth changes or storage preliminary to pupation. The larval period must be considered as a nutrition unit. In the case of larvae on 2 per cent yeast, the insect obtains in 11.4 days sufficient food material to give

TABLE 8

Effect of larval food on size of pupae

MEDIA	LENGTH OF PUPAE	ADULTS
	mm.	
Banana mash	2.0–2.5	
2 per cent yeast	2.5–3.0	
Hot aqueous sol. unfermented banana	3.5–4.0	
Agaricus campestris	3.5–4.5	Undersized
3 per cent yeast	4.0–5.5	
4 per cent yeast	5.0–5.5	
6 per cent yeast	5.5–6.0	
12 per cent yeast	5.0–6.0	Normal size
24 per cent yeast	5.0–6.0	

the energy, wear and tear, growth and storage requirements. On 3 per cent yeast the larval period approached normal at the expense of the reserve stuffs in the pupa, for the latter is undersized. This is also true of 4 per cent yeast. On 24 per cent the larvae usually reach a size of 6.5 mm. in length on the first day and are therefore three days ahead of all larvae on media of 3 per cent yeast; still pupation occurs only 1.41 days earlier. Apparently there is a certain periodicity in the larval life, since there is a tendency for the larva to pupate after a certain length of time whether it reaches the maximum size before this period or is still undersized. The probable explanation of this phenomenon is that certain changes go on in the larva, since a metamorphosis of the nervous system and digestive glands is known to take place during this period, at a definite rate if the minimum necessary food substances are available.[13]

The pupal periods (table 5) of Drosophila, fed during larval life on different concentrations of yeast, are also plotted in figure 6. The figures show that there is no consistent variation between the pupal period of larvae which lived for a long or a short

[13] Mendel and Judson ('16) studied the proportional weights of skeletons of retarded rats and found that the skeleton grows at a normal rate in retarded individuals. On normal food the growth of retarded individuals is accelerated, but that of the skeleton is retarded till equilibrium between tissue and skeleton weight is established.

period before transforming. Therefore, the pupal period is not correlated with the length of the larval life, i.e., it has, also, a fixed periodicity.

The growth of insect larvae may be retarded on sterile fruit and then greatly accelerated by adding living yeast cells to the food. Three curves showing this effect are drawn in figure 9 and curve A 24, figure 2 has already been referred to (p. 13). The insect can be maintained for a long period of time on a minimum of protein (banana) at the same size or slowly increasing

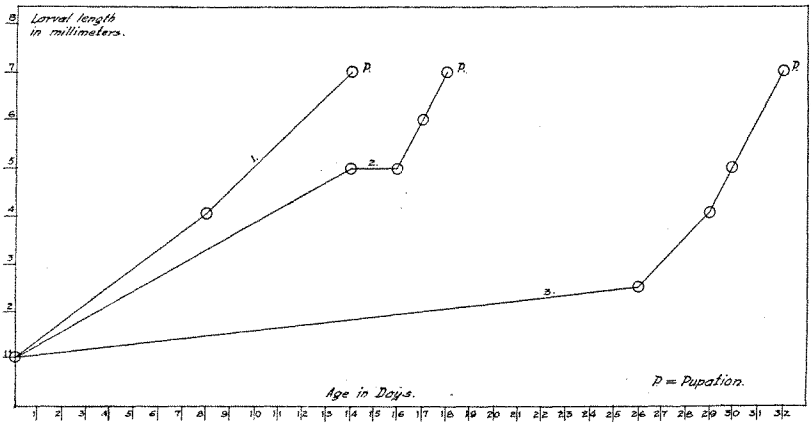


Fig. 9 The growth of retarded larvae. 1, larvae from hot aqueous extract of banana placed on 4 per cent yeast on eighth day; 2, larvae from hot aqueous extract of banana placed on 9 per cent yeast on fourteenth day; 3, larvae from cold aqueous extract of banana, living yeast introduced on twenty-sixth day.

size, after which it may be made to develop to normal size by placing on an adequate diet (6 per cent yeast). Except in one case (fig. 9, curve 3) the acceleration in the growth of retarded individuals was not above the normal rate of larvae on an adequate diet. This may be due to the fact that the length of the larva is a poor criterion of the extent of its development and that units of one day are not accurate for an animal with a normal larval period of about six days.

In this connection it is of interest that Osborne and Mendel ('14) have shown that rats can be kept for a long period at the

TABLE 9

Lengthening of life cycle by retarding larvae

AGAR MEDIA	EGG PERIOD	LARVAL PERIOD	PUPAL PERIOD	LIFE-CYCLE IN DAYS
	days	*days*	*days*	
24 per cent yeast	1–3	5.14	5.18	11.32–13.32
12 per cent yeast	1–3	6.87	4.07	11.94–13.94
6 per cent yeast	1–3	6.11	4.30	11.41–13.41
4 per cent yeast	1–3	6.85	5.85	13.7 –15.7
3 per cent yeast	1–3	6.55	4.50	12.5 –14.5
Hot aqueous extract banana	1–3	28.75	5.5	35.25–37.25
		20.00	5.0	26. –28.0
Retarded on cold extract banana. Accelerated on 26th day with living yeast	1–3	32.0	5.0	38. –40.0

same body weight by underfeeding of protein or by feeding on proteins lacking in the amino-acids necessary for growth. In this way the "menopause was postponed long beyond the age at which it usually appears." The capacity to grow after adult age is not lost if the rats have been retarded ('15), since after being stunted for 100 days beyond the normal growth period, they may reach full size when put on an adequate diet. It "appears as if the preliminary stunting period lengthened the total span of their life" ('17). In March, 1916, these investigators produced evidence from their experiments that "after periods of suppression of growth, even without loss of body weight, growth may proceed at an exaggerated rate for a considerable period."

Larvae which have been retarded in their growth by an inadequate diet and then given the proper amount of yeast food develop into normal pupae and adults. The pupal period remains the same for larvae living twenty-eight to thirty-two days as for larvae that have lived five to seven days. This is shown clearly in figure 8 and table 5. Thus the total span of the life-cycle could be increased from eleven days to forty days or more by retarding the rate of larval growth. This is shown in table 9.

b. Sugar requirement of adults and larvae. The preceding experiments on synthetic media show that both adults and larvae

TABLE 10

AGAR MEDIUM	LARVAL LIFE IN DAYS	ADULT LIFE IN DAYS
Nucleoprotein + sugars + salts + water..	10 to 21 (pupate)	5 to 7+
Nucleoprotein + salts + water.....	5 to 21 (die)	1 to 4
Sugars + salts + water.....	5–(die)	5 to 10+

require sugar as food. On nucleoprotein and sugars larvae live ten to twenty-one days, grow to full size and pupate normally and adults live more than five days. On nucleoprotein and water, however, the larvae live five to twenty-one days without marked increase in size, but all adults die in one to four days. These results are shown in table 7 and figure 7. On plain sugar and inorganic salts, adults live five to ten days, while larvae die in less than five days without increasing in size. The facts are summed up in table 10.

These results show that larvae require sugars for successful pupation, but live longer on pure[14] nucleoprotein than on pure sugar. Adults, on the contrary, live longer on pure sugar than on nucleoprotein alone.

From general observations it appears that adults oviposit more readily on sugar agar than on nuleoprotein agar, but deposit most eggs and over the longest period on nucleoprotein-sugar agar. Therefore it would seem that sugars stimulate oviposition and nucleoprotein increases egg production and that both sugars and proteins are necessary for the normal activities of both larvae and adults.

c. The natural habitat of Drosophila. I have already shown by experiments that Drosophila requires sugars and protein and that these substances can be supplied in a most normal form in yeast cells, so that it is of interest to study the conditions that exist in the natural environment of the insect.

Schultze ('11) has recorded the fly as occurring in many different fermenting and decaying fruits, vegetables and fungi, fermenting tree sap, vinegar, tumors, and animals preserved in formol. Sturtevant ('16) describes a number of new species of

[14] Carbohydrates are formed as decomposition products of nucleoprotein.

Drosophila from decaying fruit, vegetables and fungi, and from feces. Henneberg ('02) suggested that the larvae probably live on the microörganisms present in these media. It is apparent that microörganisms are usually present in great abundance in the larval environment, and from my experiments it appears that they normally serve as food for the insect, since the adults are attracted by the odors of fermentation products (p. 68). Microorganisms, known to occur on the exterior of fruits, probably usually contaminate the substratum before the flies oviposit. Flies assist in establishing a suitable flora by accidentally carrying in the digestive tract or on the minute hairs of the body many yeast cells. This can be seen in cross-sections of the body (figs. 10 and 11) and has previously been shown to be the case on page 8. The yeast growth takes place more rapidly in the presence of larvae, because the latter spread the cells throughout the medium. At first sight, this would appear to throw doubt on the ability of the larvae to digest the yeast cells, but serial sections show clearly the process of disintegration. Figures 11, 12, 13, and 14, are microphotographs of successive sections through the stomadaeum, mesenteron, and proctodaeum showing the process of disintegration which takes place mainly in the middle digestive portion of the tract. However, as in the case of birds which feed upon insect eggs and seeds, many living cells pass through the alimentary canal (page 8).

In nature Drosophila larvae are usually found in a substratum suitable as a nutritive medium for microörganisms and abounding especially in yeasts. In this environment the insect has available as food both the substratum, usually fruit, and the microorganism. But why have the larvae become dependent on the latter? Three reasons immediately come to mind, viz.:

1. As all fruits which are soft enough for Drosophila larvae to live in are always infected with yeast from the air, the larvae would unavoidably ingest fungous cells with the fruit. The nutritive value of the food would soon affect the life-cycle of the insect and bring about a close adaptation to a yeast diet.

2. Larvae feed upon microörganisms and by their constant movements carry the spores throughout the substratum. In

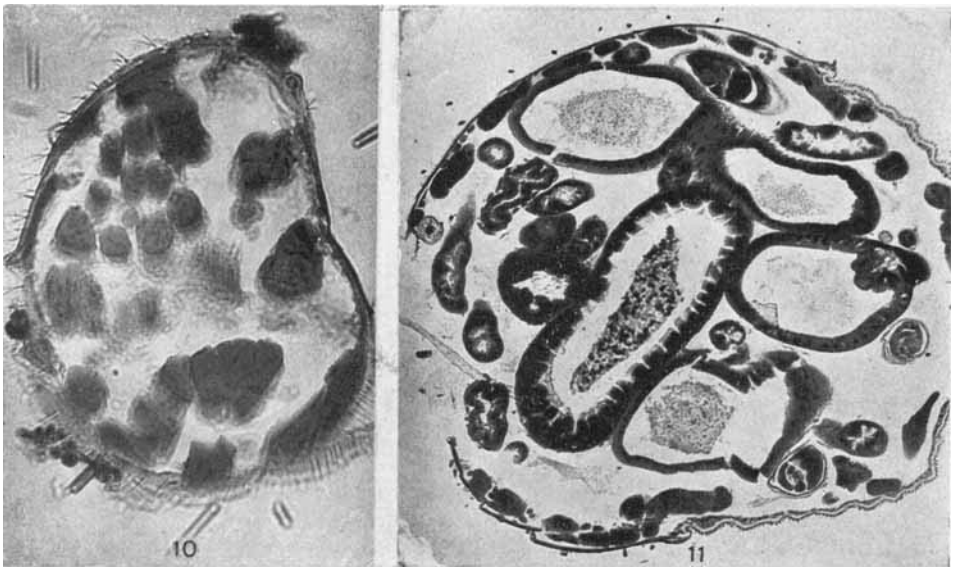
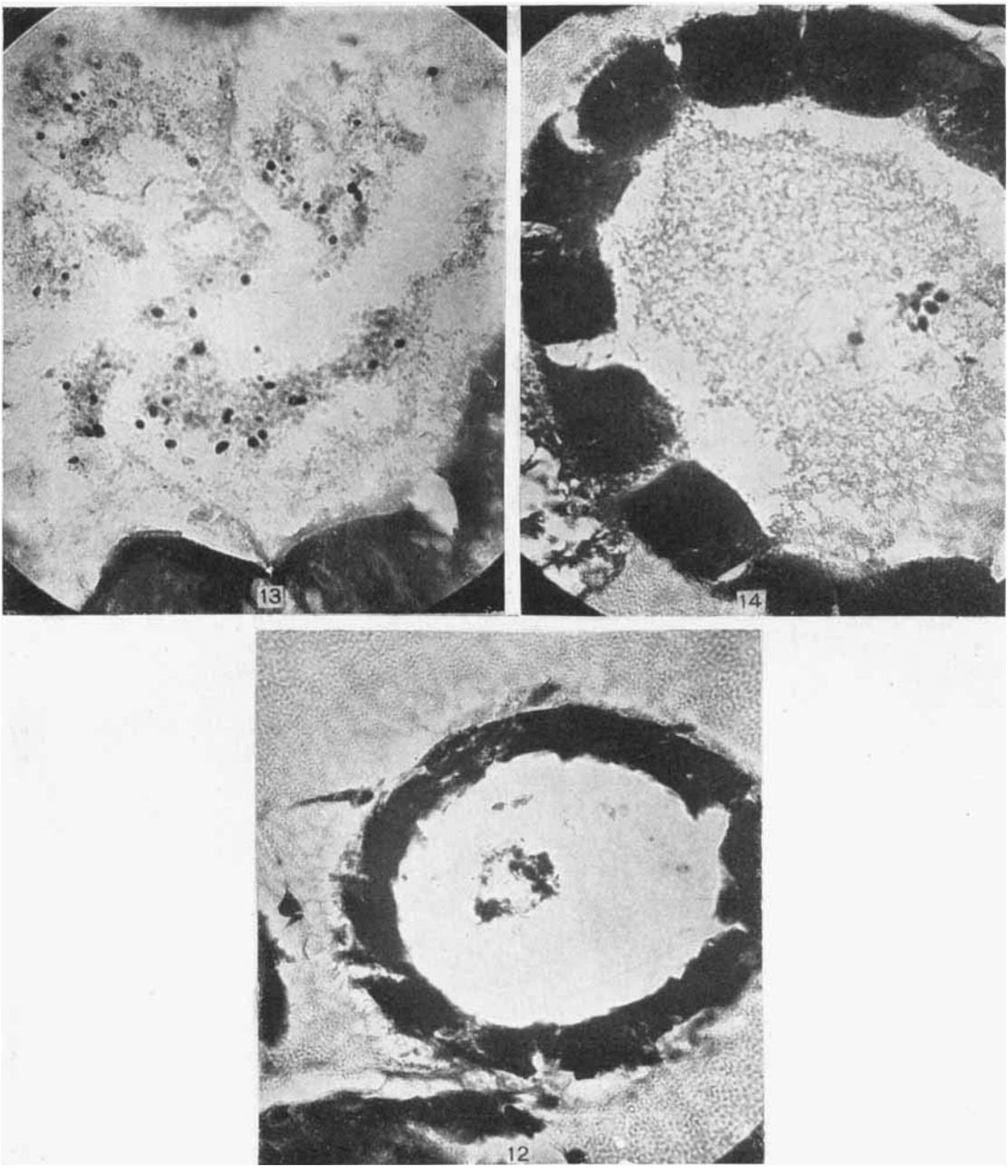


Fig. 10 Microörganisms on exterior of adult Drosophila (section through leg) (p. 32).

Fig. 11 Cross-section through abdomen of adult Drosophila showing ingested microörganisms (p. 32).



Figs. 12, 13, 14 Serial cross-sections through the digestive tract of a Drosophila larva showing t digestion of microörganisms (p. 32).

this manner yeasts often become predominant and prevent the development of destructive molds. These habits of the larvae may have come about through selective killing, for a mortality of 75 per cent was shown in cultures where the activities of the insects were suspended by low temperatures. This high mortality occurred only in cultures contaminated by fungi and was not due to the low temperature itself, but to the uncontrolled growth of the microörganisms.

3. Another explanation may be drawn from a consideration of the relative food value of the substratum and the microörganisms. The fruits or vegetables of the substratum (omitting beans, corn, spinach, etc., which are unlikely to form an appreciable number of breeding places have less than 2 per cent protein and are relatively rich in carbohydrates. Microörganisms, on the other hand, have a protein content of over 10 per cent, but are poor in carbohydrates.[15] This is shown in table 11.

The protein content of yeast cells is readily available, as the enclosing cell membrane has been shown to be closely allied to pectin (Casagrandi). Salkowski ('94) finds that the membrane is composed of two layers, one of which forms on hydrolysis *d*-glucose and the other glucose and mannose. The membranes are readily destroyed by the digestive juices, as yeast is extensively used as protein food for farm animals and even for man (Salomon, '16). The components of the yeast nucleoprotein include almost every known monoamino-acid cleavage product,

[15] Kappes' ('89) analyses of Micrococcus prodigiosus scraped from the surface of solid media gave on an average: Water, 85.5 per cent, and dry matter, 14.5 per cent, the latter having a percentage composition of protein (N × 6.25) of 71.2 per cent, i.e., 10.3 per cent for the whole cell. Nucleoprotein has been separated from cholera, bubonic plague, anthrax, and diphtheria bacilli and from B. pyocyaneum megaterium and Staphylococcus pyogenes aureus (according to Benecke, '12) and from yeasts by Hoppe-Seyler ('71), Kossel ('79), and Stutzer ('82).

Clautrian ('95) showed the presence of glycogen in dried Boletus edulis (20 per cent), Amanita muscaria (14 per cent) and yeast (31 per cent). In 1866 Hoppe-Seyler found 0.25 gram lecithin and 0.44 gram cholesterin in 81 grams of dry yeast, and later Naegli and Loew ('78) found 5 per cent fat (stearic and palmitic acid) in the yeast cell. Yeast gum (mannan) makes up 6 to 7 per cent of yeast (by dry weight), according to Salkowski ('94). Later the following gums were isolated from different yeasts, viz., mucin, dextran, laevulan, mannan, arabin and galactane (Lafar, '03).

TABLE 11

FOOD MATERIAL	WATER	PROTEIN	FAT	CARBOHYDRATES	ASH	AUTHORITY
Fruits	85.9–37.5	1.0–0.2	1.2–0.0	14.4–2.7	0.6–0.1	Atwater ('17)
Vegetables	94.3–44.2	1.8–0.4	0.6–0.1	21.9–2.2	3.2–0.4	Atwater ('17)
		8.65				Stutzer ('82)
Yeast	65.1	11.7	0.4	21.0[1]	1.8	Atwater ('02)
B. prodigiosus	85.45	10.33	0.70		1.75	Kappes ('89)
Putrefactive bacteria	83.42	13.96	1.00		0.78	Nencki and Scheffer ('80)
Mushroom	88.1	3.5	0.4	6.8	1.2	Atwater ('17)

[1] Probably largely starch in the compressed yeast. Glycogen makes up 31 per cent (by dry weight) and gumes 6 per cent of the yeast.

viz., glycocoll, alanine, valine, leucine, proline, phenylalanine, aspartic and glutamic acids, tyrosine, tryptophane, and probably serine and cystine (Meisenheimer, '15), and it is therefore not surprising that it forms, with sugars and salts, a complete food for Drosophila. As the preceding experiments show that Drosophila larvae require more concentrated protein than is present in the substratum, it is apparent that the habits of the insect are for this reason, adapted to the use of the richly protein microorganisms as food.

d. Function of yeast in the ecology of Drosophila. The function of yeast in a Drosophila culture is clearly defined by the following two experiments:

1. Larvae grow slowly on a weak, cold-water extract of aseptic unfermented bananas and remain at about the same size for a period five times the normal life and then die without pupating. If this culture is left open for a few minutes, in such a position as to allow a few fungous spores to fall into the medium, the larvae will increase their rate of growth and pupate in a few days.

2. Larvae on sterile 1 per cent yeast agar grow to a length of 4 mm. in twenty days and die without pupating. If the culture is inoculated with a minute quantity of yeast cells the larval period is only seven days and is followed by pupation. In both cases the yeast cells remove, by adsorption from the medium, the amino-acid molecules in their immediate neighborhood. As this goes on a steady diffusion of amino-acid molecules occurs

towards the place of lowest concentration, and thus the yeast finally adsorbs and builds up into its own protein all the amino-acids of the substratum. The yeast grows at the surface or just below it where it is carried by the larvae and therefore brings within reach of the larvae nitrogen that had been distributed throughout the medium, many parts of which could not be reached. In the experiment with banana agar the yeast not only concentrated the amino-acids of the substratum, but probably synthesized them into more complex molecules; in the second example, the living yeast merely concentrated all amino-acids at the surface of the medium without increasing their complexity. In a synthetic medium of sugars and salts, yeasts would concentrate and synthesize into protein, the ammonia of the substratum.

It has already been shown that concentrated banana permits larvae to pupate, but the rate of growth is not normal. Therefore it is apparent that while the banana is not entirely lacking in the substances necessary for complete growth, it is not as adequate to these demands as yeast or yeast nucleoprotein. Therefore we may conclude that the function of yeast in the ecology of Drosophila larvae is to concentrate at the surface and synthesize the ammonia[16] and aminoacids of the substratum into nucleoprotein, which fills the protein requirements of the larvae.

e. Literature on the food of Drosophila. Valuable contributions to our knowledge of the food relations of microörganisms to insects have been made by Delcourt and Guyénot. These authors reported in 1910 experiments with Drosophila in which they showed that the larvae could be reared on a potato medium free from all microörganisms except yeast or a complex of yeast and acetic-acid bacilli. Microscopical examination showed the yeast cells in the digestive tract in all stages of digestion. This paper was followed by a second in 1913 (a) in which the authors determined whether the insect fed on the products of the yeast's chemical activities or upon the yeast cell itself. In order to obtain this information, it was necessary to operate with larvae

[16] Yeast can also synthesize protein from a urate source of nitrogen.

that were sterile or with which only a single species of microörganism was associated. This was accomplished by means of an ingenious method for the aseptic transfer of adults from one flask to another and by the use of various media adverse to different species of molds and bacteria. This method of sterilizing the larvae is much less direct and requires more time than my method of sterilizing pupae with alcohol. The flies were finally found to be sterile except for the presence of yeast cells and these were eliminated by a rapid transfer of females from bottle to bottle, thus permitting aseptic oviposition in a few cases. The sterile larvae which emerged were then fed on a medium of potato and dead baker's yeast or dead baker's yeast, water, and cotton. The authors at this time made no definite statement about the function of the microörganisms, but left that for later papers. In 1913 (a) Guyénot reported that he had been able to raise fourteen generations of Drosophila in the absence of living organisms. The larvae were reared equally well on potato and living yeast, potato and dead yeast, and on dead yeast alone, but did not grow normally on sterile potato. Guyénot ('13 b) therefore concluded that in nature the larvae nourish themselves principally on living yeast and other microörganisms.

The work of Delcourt and Guyénot was unknown to me until after I arrived at similar conclusions[17] by different methods. The experiments with Drosophila as reported above are therefore in part an independent corroboration of the work of these authors.

Loeb ('15) reared Drosophila on a medium of salts and sugars with ammonium tartrate as the only source of nitrogen and therefore concluded that this insect has as great synthetic power as bacteria. Later ('16) he pointed out that yeasts may have been intermediate in the synthesis of protein, and in a third paper (Loeb and Northrop, '16 b) showed that yeasts serve as food for Drosophila and are required for the growth of the larvae.[18] These authors were unable to isolate the substance in the yeast

[17] My experiments extended over the entire period between May 1, 1916, and June 1, 1917, and were partially published in three papers (see Bibliography).

[18] Eggs were sterilized by washing in 0.1 per cent $HgCl_2$ for six to seven minutes.

on which larval growth depended, but found that the microörganism when extracted with hot alcohol could no longer serve as food for the insect. The addition of those special substances necessary to higher animals did not take the place of the substance extracted from yeast. The insects could not be reared on the normal salts, sugars, and amino-acids or proteins sufficient for higher animals, viz., cane-sugar, $MgSO_4$, NaCl, and $CaCl_2$, with casein, edestin, egg albumin, or a mixture of leucine, alanine, glycine, asparagine, tyrosine, tryptophane, and histidine, or with milk. Twelve successive generations of the flies were raised aseptically on yeast, water, and citric acid. It should also be mentioned that Loeb and Northrop raised aseptic flies on aseptic unfermented banana, but were unable to secure a second generation from them even after feeding the adults on yeast, as both sexes were sexually sterile.

My experiments show that Drosophila can be reared normally on yeast nucleoprotein, sugars, and salts, therefore any 'special substance' required by the larvae must be present in this mixture.

As previously mentioned, I have been able to rear sterile larvae on sterile hot aqueous extract of banana agar and obtain adults which appeared to be sexually sterile, as they did not oviposit on the banana during six days (the usual preoviposition period being twenty-four to forty-eight hours), but when half of the number were transferred to an aseptic 4 per cent yeast-agar medium, the females oviposited in one to three days. The larvae that emerged reached a length of 5 mm. in three days; the females remaining on the banana did not oviposit. Guyénot ('13, b) has explained this as a nutritional phenomenon. He observed that normal females from yeast-fed larvae placed upon a poor food, such as carrot, after a few days deposit eggs which though fertilized no longer develop to maturity, but die as partially developed embryos. If the same female recopulates after a period, it at first deposits normal fertile eggs, then abnormal fertilized eggs, and finally unfertilized eggs. The following experiments of Guyénot's ('13 d) will serve further to illustrate this point. He reared adults from aseptic larvae fed on sterile potato, but found that most of them were almost sexually sterile. Oviposition did not

begin till the females were seven to twelve days old (normal period thirty-six hours) and the number of eggs was 117instead of the normal 576 (24 per day). Only five larvae emerged, 49 embryos died owing to deficiency of the sperm, and 63 eggs were unfertilized. Anatomical examination by the author ('13 e) showed that only 20 to 40 eggs are normally formed in the body of the female at the time of emergence from the pupa. These are deposited in forty-eight hours, and after that all the stored material in the eggs, normally 24 per day, must be derived from the body and food of the insect. The effect of the food of the adult upon fecundity is very marked, thus 'non-fertile' sister adults from potato-fed larvae were placed, 1) on potato, where they laid one egg per day for 7 to 13 days and, 2) on potato and yeast, where they laid 10 to 15 eggs per day after 5 days and then 24 eggs per day. The converse experiment was to place sister adults raised from larvae fed on potato and yeast on 1) potato and yeast, where after 24 hours, 20 to 27 eggs per days were deposited for 10 to 17 days and, 2) on potato, where after 24 hours 20 to 27 eggs were deposited for 3 days and after that but 1 egg per day. These experiments all account for the death in the embryonic stage of eggs of a normal female, but the following experiment shows clearly that it is due to resorption by the female of the sperm cells in the bursa copulatrix (Guyénot, '13 b). 1) Adults from larvae reared on potato when placed on yeast laid from the 4th to 15th day 300 normal eggs, on potato after 7 to 13 days, 2 to 3 fertile eggs, later 20 eggs which died without hatching although fertilized, and finally 30 unfertilized eggs. 2) Adults from larvae raised on yeast, when placed on yeast deposited 24 eggs per day after 36 hours, and on potato, behaved the same as adult bred on potato, but the effect was slightly postponed.

The foregoing considerations show that the fertility of adults is a question of gross nutritional requirement and that it is difficult to interpret the yeast requirement in these cases, as a need of special substances. This is especially true since the accessory factors or vitamines which have been studied by Funk ('11), Osborne and Mendel ('13), Hopkins ('12), and others are necessary only in extremely minute quantities and are not used up in a

short period, as would have to be assumed in the case of the female Drosophila.

Guyénot ('17) has summed up all this work in a thesis and has added some experiments concerning the exact constituents necessary for a synthetic diet for Drosophila. In this he is successful to the extent that with one exception the components of an adequate diet are discovered. These are peptone, lecithin, inorganic salts, water, and an extract of yeast, the composition of which is unknown but appears to be a part of the yeast protein molecule. This extracted substance is most completely removed from yeast by boiling in 60 to 70 per cent alcohol and can be recognized by its solubility in boiling absolute alcohol, cold 70 per cent alcohol, and boiling and cold water. Attempts to substitute amino-acids, cleavage products of nucleoprotein, nuclein, carbohydrates, salts, organic acids, and fats for this special substance were all failures. Experiments with peptone gave best results when 4 per cent was used, but no larvae pupated unless lecithin was added, which permitted the storage of fats and pupation, but not the emergence of adults. The addition of bouillon to peptone also permitted a few abnormal pupae to be formed, but no adults emerged. Completely filtered autolyzed yeast, together with lecithin and peptone, made a complete and normal food for the insect. Liver autolyzed or extracted could be substituted for the yeast extract with equal success. The author also studied the formation of reserve fats and found that this process depended mainly on lecithin, but could go on to a slight extent at the expense of the protein derivatives in the yeast extract.

These results of Guyénot do not necessarily conflict with my own, as the special substance extracted by boiling alcohol is probably included in the nucleoprotein used in my experiment,[19] as Guyénot has pointed out. No fats were present in the yeast nucleoprotein used in my work, as I had extracted these with ether. As Guyénot found that lecithin is required, there would appear to be conflicting results in this regard, however, he also

[19] In drying, the nucleoprotein was washed with cold alcohol, but the special substance of Guyénot is not extracted unless the alcohol is boiling.

found that certain crystalline solids left on the filter after filtering autolyzed yeast could be substituted for lecithin. These crystalline substances are probably also constituents of yeast nucleoprotein.

Loeb and Northrop ('17) have recently used glucose beef agar for maintenance of larvae and adults so that the temperature coefficient of the duration of life could be determined, and Northrop ('17 a) has shown that the total duration of the life of Drosophila can be increased by retarding the growth of the larvae, as the pupal and imaginal periods do not seem to change with the increased larval life. These results are entirely comparable to those given on page 30. Northrop ('17 b) describes experiments which have led him to the conclusion that yeast supplies a special substance necessary for the growth of Drosophila larvae. This author finds that banana, casein, and sugar supplement yeast as a food for larvae and permit the development of a larger number of adults than could take place on yeast alone. The optimum mixture contained 33 per cent yeast, and as the amount of yeast decreased the number of adults reared became less and growth of larvae slower until at a proportion of yeast of 1:128 the growth of larvae became abnormal. Kidney, liver, and pancreas of dog were adequate foods for larvae, but spleen, heart muscle, muscle, blood, adrenal, and thyroid were not a complete diet for the insect. The author concludes that the special substance required for growth cannot be obtained from protein or carbohydrates. From my experiments I have evidence (p. 14) that banana and sugar have food value for Drosophila larvae, and to this extent my results are in accord with Northrop's, however, since the insects can develop normally on yeast nucleoprotein, sugars, and salts it seems probable that the special substances required for the growth of Drosophila are included in nucleoprotein.

In summing up the results of my experiment I conclude: 1. Drosophila normally feeds on fermenting fruit, obtaining a large part of its nourishment from the microörganisms, especially yeasts, which are in a loose symbiosis with the insect.

2. Dead or living yeast is a complete food for Drosophila.

3. The larvae are dependent on the nucleoprotein of yeast for special substances necessary in their growth.

4. The function of yeast in the ecology of the insect is to concentrate at the surface of the medium and to synthesize into nucleoprotein, the urates, ammonia, or amino-acids of the substratum.

2. Experiments with a sarcophagous insect

A pair of adult Acalyptrate muscid flies of the species Desmometopa m-nigrum Zett. (determination by Mr. C. W. Johnson) were received through the courtesy of Dr. W. M. Mann. They had emerged from some poorly dried snail shells, collected in the Fiji Islands, on the decaying flesh of which the larvae had fed. The adults were placed on banana and yeast agar, where the female deposited about forty eggs, most of which died owing to a thick mat of a black mucor that grew over the surface of the medium. The six larvae that emerged fed readily on the rich yeast food, and in about twenty-two days reached a size of 12 to 15 mm. in length. The black fungous mat was not destroyed and did not seem to injure the larvae. The six pupae formed were normal, and six adults emerged after three to five days and oviposited on the medium.

The usual manner of interpreting the normal feeding habits of this species would be to state that the larvae fed on decaying animal tissue. This, however, is open to doubt in view of the above experiments, and we must now consider the probability that all decaying or fermenting substrata are merely the media on which the fungus and bacterial food of the insect is growing.

3. Experiments with a coprophagous insect

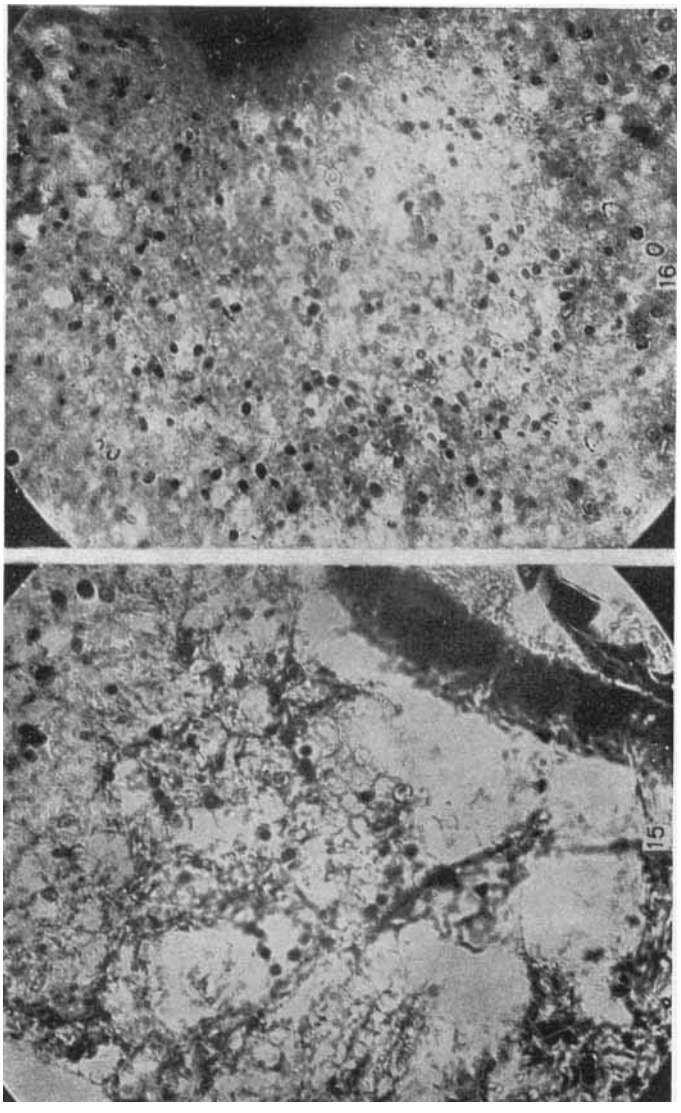
An investigation of the food of the housefly (Musca domestica) also gives support to this theory. The insects were obtained in winter by placing bran mash in the greenhouse. The mash was prepared by boiling "Educator" bran with an equal volume of water with constant stirring for twenty minutes. It was placed in a large porcelain dish in the hothouse, where it soon became

covered with Rhizopus nigricans and Penicillium glaucum molds. These growths were turned under each day and the bran thoroughly wetted till the mash had a sour smell and no longer became covered with the molds. Thus far no flies had deposited eggs on the mash, although large numbers of Lucilia caesar and Musca domestica fed on the moist surface. Several lumps of ammonium carbonate were then added, giving the medium an odor indistinguishable from manure. No eggs were observed, but larvae were soon found feeding just under the slightly incrusted surface. They gradually worked down in the medium as they became larger and the upper portions of the mash were dried out or the nutritional substances "burned" out by the fungus due to over-aëration. When this same medium was used a second time, larvae were seen to penetrate into hard lumps of the bran which still retained a visible white powdery appearance of fungous mycelia.

Two-day-old (5-mm.) larvae were transferred to bouillon yeast, banana and yeast, bran and Pasteur's agar media. The larval life was as follows:

Bouillon agar	5 mm. to pupation	13.5 days
Pasteur's agar	5 mm. to death	2.0 days
Bran agar	5 mm. to imago	8.0 days
Yeast agar	5 mm. to pupation	4.0 days
Banana and yeast agar	5 mm. to pupation	4.0 days
Bran mash	5 mm. to pupation	13–21.0 days

The larvae showed signs of great disturbance when placed in the Pasteur's medium where the sugars seemed to act as a poison to them. On bouillon agar the larvae were not very successful in completing growth and usually formed abnormal pupae; on the other agar media growth was more rapid than in the bran mash. This was due to the luxuriant growth of yeast, molds, and bacteria which the bran, banana and yeast agar supported and which served as food for the larvae. Sections through the larvae from the bran mash showed a complete absence of all material except bacteria, fungous spores, and yeast cells in the digestive tract. The microphotographs in figures 15 and 16 show the process of digestion of the microörganisms and leave no doubt that they



Figs. 15, 16 Serial cross-sections through the digestive tract of a Musca domestica larva showing the digestion of fungus spores (p. 44).

serve as food for the insect. Furthermore, the larvae are never found in any materials that are not infected with microörganisms and are in a process of decomposition or fermentation, and it is doubtful that the larvae find in these substrata nutritional substances which will be of great value to them, for the food materials are rapidly changed to decomposition products which are either absorbed by microörganisms or are too simple in composition to be available for the insect.[20] The real function of the microörganism is to synthesize protein from ammonia, urates, etc. Female flies seldom deposit eggs in substances which do not have the odor of ammonia, which as products of the action of yeasts, molds, and bacteria indicate the presence of the fungous foods of the larvae.

An attempt was made to sterilize Musca domestica pupae by washing in 85 per cent alcohol or in 85 per cent alcohol saturated with $HgCl_2$ for periods of from five to forty minutes, but in each of the 200 pupae used in the experiment, Aspergillus or Penicillium mold developed around the pupa. It would seem that the molds are carried within the pupae, although this is not definitely proved. In this connection the following quotation from Bogdanow ('08 b, p. 199) is of interest:

> Wenn Calliphoralarven bei Anwesenheit von Bakterien nur von Albumosenlösung ernährt werden können, so können die Larven von Musca domestica umgekehrt mit Stärkekleister oder mit Gelatine ohne Zusatz anderer Stoffe gefüttert werden, aber, soviel ich beobachtet habe, nur dann, wenn Schimmelpilze und Bakterien da sind.

Prowazek ('04) has found that Apiculatus fungi are usually present in the intestine of Musca and Sarcophaga larvae. A number of experiments have been performed to determine whether or not the pathogenic bacteria, with which housefly larvae become contaminated in their natural habitat, survive in the fly during the pupal period. Most of the results (Graham-Smith, '13) show that only such spore-forming bacteria as anthrax pass through the pupa alive. Tebbutt ('13) in this connection raised houseflies on agar, a little human blood, and living bacillus dysen-

[20] See page 48.

teriae (type 'Y') and B. typhosus. The fly eggs were sterilized by washing in 3 per cent lysol for two to three minutes. The pupal contents were plated and found usually to be sterile. Tebbutt does not mention the fact that the larvae probably obtained their nourishment from the bacteria.

From these experiments it appears very probable that the larvae of Musca domestica feed on microörganisms and are associated with them in the same manner as Drosophila and yeasts.

4. Experiments with a mycetophagous insect, Sciara, and a mite, Tyroglyphus, living in decaying wood

a. Experiments with Sciara. Through the courtesy of Mr. A. M. Wilcox who turned the material over to me, I was enabled to work on another fungus-eating insect found in twigs of the mountain ash apparently affected by 'black knot' or some dry black-rot disease. Under the bark and in the cambium of the wood slender white worm-like larvae, 12 to 15 mm. long, with a shining black head could be seen working in a glossy gelatinous sheath which they appeared to spin or secrete. As determined by Mr. C. W. Johnson, the larvae proved to belong to a species of Sciara, a genus of fungus gnats which feed in decaying vegetable matter and are pests on cultivated mushrooms.

The larvae were transferred to a medium of bran agar which they infected with a mucor, a Gleocladium, and a Fusarium. The larvae moved on the surface of the agar through the field of vertical sporophores with their black globular sporangia overhead. Occasionally one would raise its head and sieze a sporangium between its mandibles. The disintegrating sporangia could also be seen in the digestive tracts of the semitransparent larvae. The mandibles are peculiarly fitted for such feeding, as they are quadrate in form and having three large and several small interlocking teeth. The flat surface which the mandibles form would make it impossible to seize any structure not raised above the surface of the substratum. The larvae are also very fond of the mucilaginous secretions or exudations which appear as brilliant globules on the sporophores or sporangium walls and as a sheath around the larvae.

As mentioned above, the larvae appear to move in a gelatinous sheath over the wood. This habit has been observed in a number of fungus-eating Diptera and has been described by many authors as a secretion of the larvae. The following extract from Malloch ('17) will illustrate the present interpretation of the habit:

> Nearly all of the larvae (Mycetophilidae) spin webs in the galleries they make in their food; in the case of species that live externally upon fungi the web is slimy, rather loose and irregular. I have paid particular attention to some species I have reared, and find that the larvae of this group do not pass over the threads but through them as in a tube, the body enclosed except anteriorly. The threads are slimy in nature and the presence of the larvae may be detected by the glittering surface of the fungus which appears as if a slug had crawled over it (p. 250).

My larvae, feeding on mold in bran media, could be observed very closely under the binocular microscope. It was seen that in passing through the 'field' of fungus the larvae usually took a certain course, thus forming a 'runway' similar to that made by a rabbit in high grass. The sporangia of mucors are converted into a mucilaginous mass when the spores are discharged and the sporophores also secrete a sticky fluid, both of which stick to the surface of the larvae as they pass through the fungous growth. Thus a shining gelatinous sheath is formed through which the larvae pass. When moving over its course the larva 'flows' along in a large drop of liquid which completely surrounds the insect and assumes the same form. The surrounding drop, if stained with eosine and examined under the 1.6 mm. objective, proves to be a mass of spores of various kinds all arranged as though embedded in a clear unstained substance. A larva will often reach out and eat a portion of the surrounding drop of another larva that is passing. The sheath when stained shows a mass of mycelia growing from the spores embedded in the gelatinous matrix of fungous mucilage.

Brues ('02) has described the 'web' of Neoglaphyroptera opima Loew. and believes that it is spun by the larva which is found under the bark of fallen trees. As the insect is quickly killed by evaporation, he believes the web to be a protection against this danger. The larva was described as at times moving its head towards the web as though eating it.

From the foregoing observations on Sciarid larvae, it is apparent that they do not spin the web or secrete the gelatinous tube which surrounds them, but merely become covered with the exudations and spores of the fungi on which they live, and these spores, exudations, and hyphae serve as food for the insect. Upon pupation the enveloping drop of mucilaginous material surrounds the last larval skin and the pupa forming a cocoon of spores from which mycelia grow out. If the larvae are placed on a smooth paper under the binocular they are unable to move their long footless body. The mandibles with their flat surface cannot grasp the small particles of fiber which do not stand out above the surface. If a drop of water is placed upon the larva it immediately moves about actively by means of a ripple of circular contraction which starts at the posterior end and rolls a collar of integument over the anterior end. The anterior end is then protracted and the process repeated. It is apparent that in such a method of locomotion an enveloping fluid of high surface tension would be of great assistance. The function of the 'accidentally' accumulated mucilaginous envelope is twofold, first, to serve as a protection against evaporation and, second, to assist in locomotion.

The larval period is about twelve days and the pupal period four days. Adults are very active and run about rapidly, the male when in pursuit of the female flapping its wings vigorously. Adults may be seen to eject a hard white gelatinous body composed of fungus hyphae, etc. The adults and pupae seem much more immune to fungus attack than Drosophila. In the pupal stage this protection may be due to the complete envelope of gelatinous substance. The female deposits several separate piles of light yellow spherical eggs on the medium. These likewise seem to be immune to fungus injury as the mold often completely envelops them without causing death. The development and movement of the embryo can be observed through the egg which hatches in three to four days.

An attempt was made to sterilize the eggs and pupae, but death always resulted, probably owing to the soft exterior of these stages. The insects grew equally well on bran agar, yeast agar, and banana agar, feeding upon the luxuriant fungous growth always present.

The consideration of main interest in the present paper is the peculiar relation of substratum, microörganism, and insect which again finds an example in the food of this animal. It is is well known that molds contain enzymes capable of dissolving cellulose and hemicellulose, i.e., celluloses and cytases, which enable them to extend hyphae throughout the woody tissue of trees, etc., thus extracting all the nutritional substances. The nitrogenous natter is largely stored in the form of protein in the spores of the fungus, whereas the excess of carbohydrates may be excreted in the sticky drops of the sporophores. The insect feeding on the fungus, the wall of which it can dissolve, derives the benefit of the enzyme activities of the mold. If a section is made through larvae which have been feeding in the wood, it is seen that the great quantities of wood that pass through the digestive tract remain unchanged in structure. On closer examination fungous growths of an exobasidiomycete can be seen in the tissue cells (fig. 17). These fungi are dissolved out by the insect digestive enzymes and serve as food. The wood eaten by Sciarid larvae is therefore merely the substratum in which the fungous food material is embedded. This type of relationship is quite common among 'wood-eating' insects and is quite comparable to the symbiosis of Drosophila and yeast.

b. Experiments with a mycetophagous mite living in decaying wood. A mite of the genus Tyroglyphus (determination by Mr. N. Banks) was also found on decayed mountain-ash twigs and bred upon bran agar like the Dipterous larvae described above. Five mites added to the tube climbed about on the thick growth of fungus, apparently eating the spores in the muclaginous sporangia. As the mites rapidly multiplied the growth of the molds was checked and they were cleaned off the surface till only a few blisters or pustules of Fusaria remained. The mites could be seen to feed in large numbers at the edge of these pustules which served as food for two months allowing the mites to increase enormously in size and number. Many of this same genus of mites are known to feed on cheese, ham (on which powdery molds grow), and dry molds of various kinds, and manure, decaying fungi, and vegetable refuse are always inhabited by mites

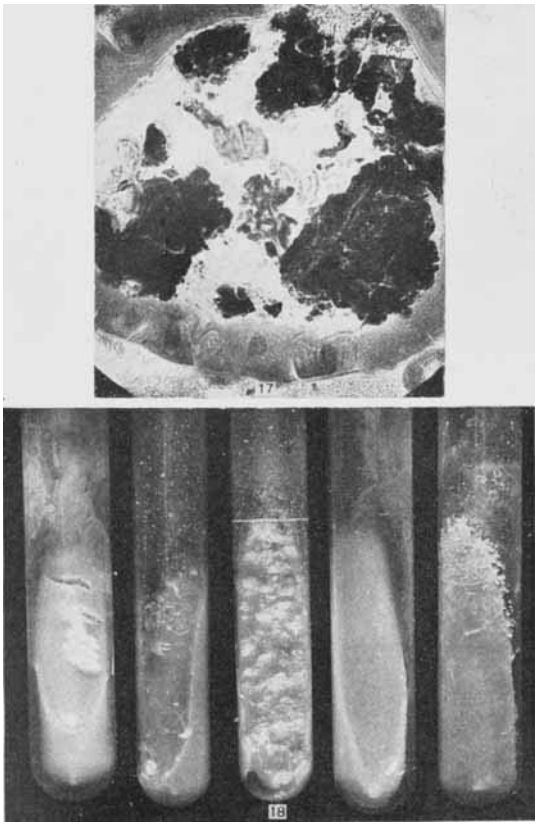


Fig. 17 Cross-section through the digestive tract of a Sciara larva showing the fungus mycelium on ingested woody tissue (p. 50).

Fig. 18 Agar Drosophila cultures (from left to right). 1st tube inoculated from second tube. 2nd tube, large larvae and pupae on non-sterile banana. 3d tube, small larvae of same age as 2 on sterile banana. 4th tube, inoculated from 3, showing its sterility. 5th tube, large larvae and pupae on 6 per cent dead yeast of same age as 2 and 3 (p. 11).

of various species. It is quite probable that mites inhabiting decaying and fermenting material, feed largely on the microörganisms present.

c. Association of wood-eating insects with fungi. The reason for the association of Sciara and Tyroglyphus with fungi is probably because of the indigestibility of the cellulose walls of the wood and the small amount of protein contained in them. The composition of wood varies with the season and with age, species, location, and tissue, so that it is difficult to make any general statement. Haberlandt ('15) has recently studied the digestibility of wood and concludes that unless the cellulose is destroyed or changed, wood has little food value for mammals, as the nutrient substances are inaccessible. Birch-wood was found to have the highest nutritive value, giving the following analysis: water, 4.56; nitrogen, 0.108 (protein, 0.675); ether extract, 0.45; nitrogen-free extract, 61.56; crude fiber, 32.2, and ash, 0.46. In general it may be said that, except in the living phloem and cambium, the protein content of wood is extremely low. Carbohydrates are usually abundant and wood is therefore used in the manufacture of sugar, some processes yielding as high as 25 per cent. These compounds are probably quite inaccessible to insects because of their chemical and physical nature, but are readily dissolved and converted by fungi due to their notable enzyme activities.

The low nutritive value of wood causes the insect to either lengthen its life-cycle so as to be able to extract a greater amount of wood or it leads to association with microörganisms either as food or as symbionts. In the first class belong the large majority of heartwood-boring Lepidoptera, Coleoptera, and Hymenoptera, for example, the moth larvae Zeuzera pyrina and Sesia apiformis with a two-year life-cycle; the larvae of the beetle Saperda populnea with a two-year, Elaterid larvae with a three-year, Melolontha vulgaris with a four-year life-cycle, and Sirex (Hymenoptera) with a larval period of one year. The next step which may lead to the habits of the ambrosia beetles and termites might be the reingestion of material already passed through the digestive tract as described by Escherich ('95) for the beetles of the family Ipidae (Bostrychidae) which he believes to be adopted

for the purpose of extracting all the nutriment possible from the food already comminuted. This would lead us to the case of the Cecidomyiid, Asphondylia prunorum, which was studied by Neger ('08 a, b; '10). The adult deposits with the egg on the prune tree a mass of fungous spores and mycelia which serve as food for the larva and finally grow on the tissue of the gall formed. The fungus itself is not concerned in the gall formation, but merely serves as food for the gall inhabitant. Upon the emergence of the adult insect the fungus breaks through the gall and can be seen as a white growth from the outside. The fungus, a Macrophoma species, is very similar to the fungus fed upon by the wood-boring beetles (Xyleborus, Xyloterus, etc.) and has never been found elsewhere; the galls have therefore been called 'ambrosia-gallen.'

At the pinnacle of this development may be placed the ambrosia beetles and termites. Schmiedberger ('36) first gave the name 'ambrosia' to a protein-containing white substance which he found to be the food of the insect rather than the chips of wood cut by them. This was made certain by the observations of Hartig ('44) and it was also decided that the white substance was a fungus which grew on the wood cuttings. The subject was further studied by Hubbard ('97) in America and Neger ('07) in Germany. Though these two investigators do not agree in all their observations, they have made certain that different species of fungi are associated with different species of beetles and that these associations are constant for the same species in spite of changes of host plants or parts of plants eaten (Xyleborus saxisenii). The fungus is independent of the food plant, but dependent on the products of the insect. Hubbard maintains that the female consciously carries the spores of the fungus to the new gallery and sows them. Neger, however, believes that the spores become attached to the highly sculptured wing cases of the female as it leaves the larval gallery, the walls of which are coated with the fungus. The fungus that grows on the walls of the galleries is different for different beetles, but in general is composed of either a chain of round cells which are assembled in an irregular heap or of upright threads with a round corpuscular cell on the

tip. The latter condition is termed conidial by Hubbard. Escherich observed that these conidia served as food for the beetles and their larvae. As a certain degree of moisture is required by the fungus, the insects never select dried-out trees for their galleries, but always bore in wood which retains some sap. Other species of ambrosia beetles (Corthylus) are able to live in sapwood in the absence of the fungus, perhaps because of the abundance of protein in that region. The fungus is usually propagated in a little bed of chips, prepared by the female, in which the egg is deposited. In other species the woody tissue, after passing through the digestive tract of the larvae, has a yellow mustard color. In this condition it is plastered on the walls of the gallery and serves as a medium on which fungus grows. Undoubtedly, under these conditions the fungus may be considered as a chemical collaborator digesting food indigestible to the insect and furnishing it in a tender luscious form to the larvae.

Hedgcock ('06) studied fungi from various species of Ambrosia beetles and was able to refer them to the wood-bluing (Ceratostomella), wood-blackening and -browning (Grophium, Hormodendron, and Hormiscium) and to the wood-reddening (Penicillium and Fusarium fungi. According to Escherich and Neger, the fungus in the absence of the larvae may assume a slightly different form. Neger has also found fungi in the galleries of Cerambyx and Tetropium luridum, but is unable to decide whether or not it serves as food.

The fungus-growing habits of the white ants or termites are principally known through the work of Hagen ('60), Holte ('99), Haviland ('02), Trägardt ('04), Doflein ('05, '06), Petch ('06), and many others. These wood-eating insects build subterranean nests, the ground which they excavate being placed in a pile which itself is later used to form chambers. Shafts may be left in this superstructure with an outer chimney; these are used as permanent scaffolds and have little effect on the ventilation of the nest. The fungus gardens are either on the floor or suspended from the ceilings of the chambers, and consist of a mass of comminuted woody tissue which has passed through the digestive tracts of the workers and is then built into the comb. The fun-

gus which grows on this comb (as in the case of ambrosia beetles, really upon food cut by the insect, but largely indigestible to them, is described by Petch ('06) as follows:

> The mycelium on the comb bears small, white, stalked or almost sessile 'spheres.' These consist of branching hyphae bearing either spherical or oval cells. The spherical cells do not germinate. The oval cells germinate readily but it has not been possible to reproduce the 'spheres' from them. When the comb is old an agaric grows from it. This agaric appears in two forms, one of which has been assigned by various mycologists to Lentinus, Collybia, Pluteus, Pholiota, and Flammula, and the other to Armillaria. It develops in a cartilaginous, almost gelatinous, universal veil and is a modified Volvaria. Other fungi which grow on combs removed from the nest include Mucor, Thamidium, Cephalosporium, and Peziza. As these are not found in the nest, though some of them are capable of development underground, it is probable that the termites 'weed out' foreign fungi from the cultivation of the comb. *The comb material is probably sterilized by its passage through the alimentary canal.*[21] That the spheres form the food of the termites is probable, as in the case of the leaf-cutting ants; neither case can be considered definitely proved. *Termes redemanni* and *T. obscuriceps* undoubtedly *prefer fungi, or wood which has been attacked by fungi* . . . . It is most probable that the 'spheres' in the termite comb and the 'Kohlrabihäufchen' of the leaf-cutting ants investigated by Möller are parts of a normal mycelium, and that their shape is modified by the insects only in a very slight degree, if at all . . . . The available evidence appears to show that the 'spheres' are part of the mycelium of the Volvaria, but it has not been possible to connect these forms experimentally.

The fungus gardens are found in all chambers except the royal chamber; here the queen lies in state and is fed (Doflein) with a concentrated and easily assimilated food consisting of mycelial spherules by the workers. The larvae, according to Petch, do not show the presence of any spherules in the digestive tract, but may be fed on some regurgitated or predigested food furnished by the workers, which in turn feed on decaying wood. It is of interest that the queen termite is the only known adult insect which increases in size. The queen is usually the center of a pool of fatty secretions on which the workers feed with great satisfaction.

[21] My italics.

Undoubtedly these complicated habits have come about by taking advantage of the enzymatic and possibly synthetic power of the microörganisms. The type of association is the same as with Drosophila, but is further complicated by the physical properties of the wood and the habits of the insect.

Some insects that feed on wood are apparently not associated with any microörganisms, for the burrow does not appear to be discolored. However, as a general thing, larvae are in symbiosis with some microörganism when boring in woody tissue. When feeding on leaves larvae are often completely or reasonably sterile, and this is what would be expected from the foregoing assumptions, for the tissue of the leaves is very soft and readily digested.

Portier ('05) has thrown some light on this subject by his studies on the caterpillar of Nepticula, a small lepidopterous insect that feeds in the parenchyma tissue of rose leaves. The eggs are deposited on the leaf, which he supposes is sterilized by the sun's rays, and the larva bores directly down into the leaf, sealing up the entrance; the feces are not thrown out as in the case of Tischeria. The exterior of the leaves were sterilized by Portier and the whole leaf, with the excavation cut open, was covered with bacterial media. The fifteen cases investigated were perfectly sterile[22] whereas all cases of species which throw out the feces (Lithocolletis and Tischeria) were contaminated with bacteria and fungi, especially Aspergillus niger. Later ('11 a) Nonagria typhae larvae, that live in the trunk of Typha latifolia, were investigated, and it was found that they were associated with a pseudobacillus present in all tissues of the body, having passed through the chitinous peritrophic membrane during ecdysis. The bacteria were in all stages of decomposition in the phagocytic cells of the blood. A second paper ('11 b) showed that in Nonagria typhae a more complex situation exists than at first described, in which two microörganisms are in symbiosis: a micrococcus and a fungus Mucidium (Isaria). This fungus must

[22] During the summer of 1917 I examined the digestive tracts of some thousand Porthetria dispar caterpillars, pupae and adults, and found microorganisms present only in pathological cases, therefore this insect is not associated with fungi.

be held in check by the larvae, since if allowed to sporulate, as occurs after death, injury would result. Portier believes the secretion of the labial glands have this function. To link these observations with the case of Nepticula, in which the larvae are aseptic throughout life, Portier ('11 c) describes the condition in Gracillus syringella, which at first feeds in an aseptic condition on the soft interior of the leaf, and then, after feeding on the exterior of the leaf and becoming associated with a digestive flora[23] capable of dissolving woody tissues, bores into the twigs, the organisms being in part absorbed by phagocytosis as food for the larvae.

Internal symbionts have also been found in a beetle, Anobium paniceum, by Karawaiew ('99) and Escherich ('00). These symbionts always occur in definite cells in the anterior end of the midgut. Karawaiew thought he recognized a vacuole in them and therefore considered them to be Flagellates. Escherich, however, studied them in hanging drops of sugar solution and determined that they were Saccharomycetes. As these yeasts always occur in the same cells and pass through the pupa into the adult, it is quite likely that they are transmitted through the egg from one generation to another. Escherich found that the number of yeast cells varied with the amount of nourishment taken in the different stages of the insects' metamorphosis, thus they were very numerous in the larva, rare in the pupa, and few in the adult. He therefore concluded that the fungi are intimately concerned with the nutrition of the insect. As the Anobiid feeds mainly on very dry house timbers, the symbiosis with a fungus could very well be of value to the insect in the extraction of food from the wood.

In general we may conclude that insects overcome the disadvantages of the chemical and mechanical composition of wood by association with microörganisms either as food or as internal symbionts.

[23] Henseval, M. (compare Biedermann) ascribes an antiseptic property to an essential oil secreted by Cossus ligniperda larvae. This oil has the property of making the wood more workable ('angreifbar').

EXTENT OF MYCETOPHAGY AMONG INSECTS

As a corollary to the foregoing conclusions we may assume that the foods of many insect larvae feeding on dead, decaying, and fermenting vegetable and animal matter are the micoörganisms which live upon the substratum in which the insects are embedded.

The extent of this habit among insects is very great, including a large number of Coleoptera and an especially large number of Diptera. This habit is usually apparent from the habitats selected by the insect, thus Metcalf ('16) lists the following habitats for the scavenger short- and long-tailed filth larvae of the flower-fly (Syrphidae), viz.: In decaying parts of trees and herbaceous plants, diseased or flowing sap, heaps of turf or soft mud containing vegetable matter, and in stagnant or putrid water, sewage, manure, or human feces. The larvae also occur as accidental body parasites, causing intestinal, nasal, auricular, and vaginal myasis. Some species serve as scavengers in the nests of termites, ants, wasps, and bees. It is apparent that microörganisms abound in all these environments, with the possible exception of the animal body. In the latter case, however, it is well known that a foul odor, indicating some bacterial action, always precedes infestation. In more normal habitats the microörganisms so completely outweigh the other nutritive materials that it is quite likely they (the bacteria) serve as food.[24]

Townsend ('93) lists the following habitats for some of the scavenger Acalyptrate muscid larvae: dung, decaying wood, under bark, plants, leaves, roots, tubers, and fungi; in salt or alkaline water and mud; urine, vinegar, sap of wounded trees; cheese and animal fats. Again in this case all habitats selected by the fly normally abound in microörganisms, and it is quite safe to assume that they (the fungi) serve as food for the insect larvae.

The great extent of the use of microörganisms as food among insects is shown in a table of the feeding habits of larval and adult Diptera. In this table I have assumed that the food of insects, that always inhabit substrata of a fermenting or decaying nature,

[24] Osborne and Mendel ('14) showed that the bacterial content of feces was 20 to 40 per cent.

GROUP	LARVAL FOOD				ADULT FOOD				
	Saprophytic fungi	Algae	Higher plants	Animals	Saprophytic fungi	Fermentation products	Higher plant juices	Nectar	Fluids of animals
Tipulidae	x	x	x		?	?		x	
Ctenophorinae	x						x	x	
Tipulinae	x							x	
Limnobiidae								x	
Cylindrotominae		x	x						
Limnobiinae	x	x			x			x	
Limnobia	x							x	
Pediciinae		x		x				x	
Limnophilinae	x	x							
Limnophila	x	x							
Eriopterinae									
Helobia	?							x	
Gnophomyia	?							x	
Hexatominae	x	x							
Trichocerinae									
Trichocera	x								
Ptychopteridae	x	x						x	
Rhyphidae	x				?	?		x	
Boletophilidae	x								
Mycetophilidae	x				?		x	x	
Leia	x								
Exechia	x							x	
Sciaridae	x							x	
Platyuridae	x							x	
Psychodidae	x	x			x	x		x	x
Blepharoceridae		x						x	x
Culididae	x			x			x	x	x
Dixidae		x						x	
Ceratopogonidae	x								x
Chironomidae	x			x				x	
Orphnephilidae	x			x					
Bibionidae	x							x	
Scatopsidae	x				x	x		x	
Simuliidae		x							x
Stratiomyiidae	x	x		x				x	
Stratiomia	x	x		x				x	
Odontomyia	x	x		x				x	
Oxycera	x	x		x					
Geosargus	x								
Microchrysa	x								
Eupachygaster			x	x					
Xylophagidae			x	x			x	x	
Coenomyiidae	x			x			x	x	
Tabanidae	x			x					x
Coniops	x			?					x

GROUP	LARVAL FOOD				ADULT FOOD				
	Saprophytic fungi	Algae	Higher plants	Animals	Saprophytic fungi	Fermentation products	Higher plant juices	Nectar	Fluids of animals
Tabanus	x			x					x
Leptidae	x			x				x	
Atherix								x	
Chrysopila	x							x	
Cyrtidae				x					
Asiloidea				x				x	x
Mydaidae				x				x	x
Asilidae				x				x	x
Dasyllis				x				x	x
Bombyliidae				x				x	
Therevidae				x				x	
Scenopinidae	?			x				x	
Empidiae	x			x				x	x
Drapetis	x								x
Dolichopodidae				x				x	x
Phoridae	x			x					
Platypezidae	x								
Bipunculidae				x					
Syrphidae	x			x				x	
Conopidae				x				x	
Psilidae			x						
Sepsidae	x				?				
Trypetidae	x		x						
Sapromyzidae	x								
Agromyzidae			x						
Geomyzidae			x						
Drosophilidae	x				x	x			
Ephydridae		x	x						
Oscinidae			x						
Phycodromidae	x								
Borboridae	x	x			x	x			
Heteromeusidae	x								
Helomyzodae	x				x				
Cordyluridae	x		x	x					
Anthomyidae	x		x	x					
Muscidae	x			x	x	x		x	x
Oestridae				x					x
Sarcophagidae	x			x	x	x			
Dexiidae				x					
Tachinidae				x					
Hippoboscidae				x					x
Streblidae				x					x
Nycteribiidae				x					x
Sciomyzidae	x	x							

is the microörganisms themselves, and to a less extent the substratum. This assumption can safely be made on the basis of the preceding experiments and the general lack of nutritive value (for insects) of many of the substrata concerned. This interpretation of the food of scavengers, etc., has never been given before to the author's knowledge except in the case of Drosophila, as mentioned above (Guyénot, Loeb, Schulze, Henneberg). The data in the table are mainly derived from Malloch ('17) and Williston ('08). From a glance at the table it is apparent that a large majority of the Diptera live upon microörganisms. Therefore it might be well to include under the term Mycetophages all insects which have hitherto been termed scavengers, coprophages, etc.

Parrott, Fulton, and Gloyer ('14, '15, '16) found that tree crickets eat fungous "mycelia and spores which are unaffected by the intestinal juices."[25] This assumption is based on the fact that the spores of fungi are not digested by Oecanthus, as germination takes place from pellets of material passed through the digestive tract, and they believe

> it is possible that the spores may act as roughage and were eaten for that purpose; but it appears more plausible that the spores still retain on their surface some of the protoplasm of the ascus or pycnidium which makes them palatable ('16, p. 12).

The assumption that fungi are not digested, based as it is on the evidence that many living spores pass through the digestive tract, is not entirely justified in my opinion, since the same can be said of yeast cells in the Drosophila larvae, although in this case the plant undoubtedly serves as food.

The leaf-cutting ant is a good example of a mycetophagous insect. The fungus-growing habits of these Attiine ants should really be depicted at the same time as the habits of termites, as

[25] The wound made in tree twigs in which the eggs are placed is plugged with excrement and often becomes the seat of the development of cankers. Gloyer and Fulton ('16) give a concise account of the literature on the dissemination of plant diseases by insects. This is in large part due to the habit of insects of feeding on all available fungus and bacterial growths and in doing so getting the body covered with spores which readily remain attached to the hairs and spines on the surface of the body.

they are equally specialized. However, the origin of the habit appears to be different, as will be seen later, therefore their fungus gardening is best described at this point. Our knowledge of these forms is specially due to the work of Bates ('63), Belt ('74), Tanner ('92), and others. These ants excavate subterranean nests composed of a series of chambers connected by a vertical shaft usually ending in a crater. The fungus garden is built of the comminuted fragments of leaves, cut from trees near the nest, which have not passed through the digestive tract. The little pellets thus formed are built up into the sponge-like mass suspended from the ceiling or placed on the floors of the chambers. Caterpillar droppings are quite commonly built into the comb, which serves as a substratum for special kinds of fungi. A summary of all the literature on these ants, the termites and the ambrosia beetles, as well as some important contributions to the ethology of American species, is given by Wheeler ('07), from which I quote the following conclusions of Möller:

> All the fungus-gardens of the Atta species I have investigated, are pervaded with the same kind of mycelium, which produces the 'kohlrabi clusters' as long as the ants are cultivating the gardens. Under the influence of the ants neither free aerial hyphae nor any form of fruit are ever developed. The mycelium proliferates through the garden to the complete exclusion of any alien fungus, and the fungus garden of a nest represents in its entirety a pure culture of a single fungus. The fungus has two different forms of conidia which arise in the garden when it is removed from the influence of the ants. The hyphae have a very pronounced tendency to produce swellings or diverticula, which show several more or less peculiar and clearly differentiated variations. One of these, which has presumably reached its present form through the influence of cultivation and selection on the part of the ants, is represented by the 'kohlrabi heads.' Under artificial conditions the 'kohlrabi clusters' and 'heads' disappear and the fungus becomes a mass of bead-like conidia.

Sampaio ('94), von Ihering ('98), Goeldi ('05), and Huber ('05) have shown how the new fungus colony is started after the marriage flight. The queen deälates itself, digs a small subterranean burrow which it closes and then starts the new colony by spitting out a pellet of fungous hyphae which had been carried in the buccal pocket and depositing eggs upon it. The fungus

colony is grown upon the liquid excrement of the female, which touches small pieces of the mycelium to the tip of its gaster and then replaces them in the fungus garden. Some of the eggs are sacrificed as manure for the fungi.

Parrott and Gloyer ('16) are of the opinion that the fungus-growing habit of the Attii may have come about through the collection of caterpillar feces in which many spores accidentally eaten by the larvae are still alive. They do not give any explanation of the habit of collecting the feces, however. Wheeler ('07) points out that the gardens are usually composed of a substratum consisting largely of fecal material in the case of the ambrosia beetles and termites, and that the habit is pronounced in the lower genera of leaf-cutting ants and visible in all cases closely studied. It therefore seems probable that the food of the Attii "may have been originally grown on fecal substances" Von Ihering ('94) believes that the habit may have phylogenetically originated with ants using moldy seeds stored as food. I should suggest the possibility that the ants may originally have fed upon fungous mycelia developing on caterpillar feces from spores unkilled in their passage through the digestive tract. Such droppings might finally be carried into the nest where the fecal substratum and the moisture of the nest would soon allow the growth of a valuable crop of fungous food. Thus the fungus-growing ants developed their habits as a direct response to a valuable food supply. On the other hand, the termites developed their habits as a means of making use of an unmanageable food supply.

The examples cited above indicate that the use of microörganisms as food[26] is widespread among insects and is a direct response to the high food value of the fungous cells. The feeding habits may be grouped into three classes as follows:

1. Ingestion of microöganisms with substratum, i.e., Drosophila, Musca, Sciara, worker termites.

2. Feeding directly on microörganisms, i.e., mites, tree crickets, many adult Diptera, etc.

[26] See page 68 for case of mosquito larvae feeding on fungi.

3. Preparation of medium for microörganisms, i.e., leaf-cutting ants, termites, ambrosia beetles.

MICROÖRGANISMS AS LIQUEFIERS OF THE SUBSTRATUM

The relation of the insect, microörganism, and substratum is not always as clearly defined as in the preceding cases. Fabre ('94) studied the food of Lucilia, the green-bottle fly, and came to the conclusion that the larvae secrete a digestive fluid which allows the liquefied albuminous material to be sucked up by the insect. He placed in one tube of hard-boiled eggs a few fly eggs and left the other tube of albumin equally exposed to the air, as a check. The albumin on which the larvae emerged was soon a liquescent mass, whereas the check dried up. Guyénot ('07) reinvestigated the problem with Phormia regina Meigen and also studied the anatomy of the larvae. The mouthparts as he describes them are very similar to those of Drosophila larvae, as mentioned above. The pharynx is immediately connected with the crop of sucking stomach, a much distended flask-like structure which usually lies to one side of the oesophagus. Owing to the nature of the pseudomaxillary apparatus, the larvae are unable to eat any solid food. Fabre had supposed they secreted on the food some pepsin which liquefied the albumin. To test this theory, Guyénot ground up the larvae and made various extracts. The extracts had no effect on starch, fat, or albumin; the same was true of extracts of the salivary or gastric glands. The normal liquefaction was then studied, and it was found that the albumin was broken down to the peptone stage by a bacterium Micrococcus flavus liquefaciens (Flugge) which was always present with the larvae. The bacteria alone, without the acid of the larvae had the same effect on the albumin, but at a much slower rate. However, if they were mixed with the albumin with a sterile platinum wire the speed was as great as with the larvae present. As these bacteria were found in large quantities in the sucking stomach of the larvae, Guyénot reached the following conclusions:

I. La liquéfaction des substances albuminoides résulte d'une véritable digestion opérée par certains microbes de la putréfaction.

II. Les larves de mouches, absorbant exclusivement des aliments liquides, directment assimilables, ont un travail digestif réduit au minimum et ne produisent pas de ferments solubles en quantité appréciable.

III. Les larves accelérent la putréfaction des cadavres en favorisant la pullulation des microbes.

IV. Les larves se nourrissent aux dépens des produits du chimisme microbien; les microbes ne peuvent se développer rapidement que s'ils sont repartis en tous points par les larves. Il existe entre ces deux agents de la putréfaction une véritable symbiose (p. 369).

Guyénot does not consider that the food of the larvae may be the microörganisms themselves, and this question is still open, however, unlike the following example, the microörganisms associated with Lucilia have the function of liquefying the food material.

Bogdanow ('06) studied the similar case of Calliphora vomitoria, the flesh fly. The eggs were sterilized by washing for two one-and-one-half-minute periods in 5 : 1000 aqueous $HgCl_2$ solution and then rinsed in running sterile water and were then placed on sterile media of casein, egg albumin, etc. None of the flies obtained was sterile, but was usually associated with a micrococci which Bogdanow believed was passed through the egg. The larvae grew rapidly on casein, egg albumin, albuminoides, etc., in the presence of micrococci and a gelatin-dissolving bacterium, but the flies that emerged were few in number and very small in size, being 'starvation forms.' The larvae were later given the selection of fresh or putrid meat, showed a preference for the former, the putrid meat usually killing the larvae. Meats putrifying in the presence and absence of larvae could be distinguished by a difference in odor as the micrococcus with which the insect infects the meat liberates ammonia from proteins. The larvae grew normally on meat in the presence of a gelatin-liquefying bacterium and the micrococcus. Two factors are therefore necessary for the successful metamorphosis of the larvae the micrococcus from the egg and a gelatin liquefier from the air.

In 1908 (b) Bogdanow published a second paper on the same subject in which he showed that about 35 per cent of the eggs of the flesh fly are infested with a pure culture of micrococcus. The

other 65 per cent are sterile and can be reared on nutrient gelatin in the presence of a bacterium capable of its liquefaction. These larvae seldom result in normal-sized adults and it was not possible to raise sterile larvae on a synthetic medium of meat ash, peptone, and meat extract, acid or alkaline. Therefore Bogdanow concluded:

1. Im einfach sterilisierten Fleische wächst die Calliphoralarve gewöhnlich sehr schlecht.
2. In sterilisierten Resten der Larvennahrung wächst sie nicht besser.
3. Für die gute Larvenentwicklung sind meistens gelatineverflüssigende Bakterien oder Trypsin nötig (p. 193).

As nutrient gelatin is a highly inadequate diet for Drosophila, it is probable that the Calliphora reared on this medium obtained some of their food requirements by digesting the cells of the gelatin-liquefying bacteria which are unavoidably ingested by the larvae. As the flies were all undersized, it appears that this microörganism is at least not a complete food for the insect (as yeast is in the case of Drosophila). Therefore, Bogdanow's conclusion, that bacteria play merely the part of liquefiers in the ecology of the larvae, is largely warranted.

Wollman ('11) repeated Bogdanow's experiments and came to the conclusion that sterile Calliphora vomitora larvae can be rereared on sterile meat which has been sterilized by Tyndalizing rather than autoclaving. Bogdanow had autoclaved the meat used in his experiments, thus coagulating the proteins and making them insoluble to the larvae which when small have (according to Wollman) low proteolytic power. Though Wollman found that the larvae grew more successfully in the absence of microorganisms, as putrefactive bacteria always occur in the habitat of the fly, it is quite likely that they have some food value for the insect. Nevertheless, Calliphora, unlike Drosophila or Lucilia, grows best in the absence of all microörganisms.

ODORS ATTRACTIVE TO INSECTS

The odors which are attractive to dipterous adults are usually fermentation or decomposition products of the activity of micro-

organisms on a substratum. If we assume that the insect larvae feed upon these microörganisms, the chain of circumstantial evidence is complete. Our knowledge of the odorous substances attractive to flies has been advanced greatly by the work of Barrows ('07) and Richardson ('16a, b; '17), but these authors have never given the above interpretation to the response. Barrows studied the odors to which the adults Drosophila ampelophila (melanogaster) responds and found that the most attractive odors are those of ethyl and amyl alcohol, acetic acid, lactic acid, and acetic ether. A small amount of acetic ether, isobutyl acetate, methyl acetate, acetic or butyric acid added to ethyl alcohol greatly increased its attractiveness. "Alcohol and acetic acid are commonly found in cider vinegar, fermented cider and California sherry in per cents that are close to those which call forth the largest number of reactions in Drosophila." This odor is identical to that produced by the wine yeast Saccharomycetes ellipsoideus which I have found to cause females to deposit the largest number of eggs.

In 1916 (a) Richardson reported that he had carried on a series of experiments with odorous substances as baits for houseflies. The baits tested were placed under wire-gauze traps and were as following: Ammonium carbonate, ammonium sulphide solution, ammonium hydroxide, ethyl alcohol solution of skatol and indol, ethyl alcohol, acetic, formic, butyric and valerianic acids, hydrogen sulphide solution and carbon dioxide. "Negative results were obtained in all but the ammonium hydroxide and ammonium carbonate experiments." The ammonia was the attractive substance especially to females, which were found in the percentage of 89.2 to 7.5 of the males, although the actual percentage of sexes in the vicinity was 54 to 45.9 respectively. Valerianic and butyric acid augmented oviposition; the females, however, showed some discrimination between nutritious and non-nutritious material. In 1916 (b) a second paper gave a list of insects attracted to the ammonia, all of which spend at least part of their life in some form of animal excrement.[27]

[27] A third paper ('17) showed that aqueous solutions of carbohydrates are far less attractive than alcoholic or acetic acid solutions of such substances.

As both Drosophila and Musca domestica feed on microörganisms, it is of peculiar interest that the odors which stimulate oviposition by the female are identical to those formed by microorganisms in the substratum in which the insect normally breeds. The response of the female fruit fly to the odors of alcohol and acetic acid would indicate an instinctive response to the conditions best adapted to larval life, i.e., active fermentation. In the same way the response of the female housefly would indicate that the best conditions for housefly larvae require the presence of proteolytic (hence odors of ammonia, etc.) and fermentative (hence odor of alcohol and acetic acid) microörganisms. Richardson's results therefore give circumstantial support to my conclusion that the larvae of Musca domestica live on microörganisms.

Response to the odor of microörganisms is highly developed in the larvae of the yellow-fever mosquito, Stegomyia fasciata, as Bacot ('17) has recently shown that the eggs of this insect will remain for several months unhatched with the fully developed larva inside if the bacterial content of the surrounding water is low. The addition of foul contaminated fluid causes hatching in ten minutes. It is true that a fall of 6 to 10°F. causes some larvae to emerge, but the percentage is very low. Eggs were sterilized and transferred to sterile fluid, but if living yeast or bacteria were added they hatched immediately. Sterile autolized extract of brewer's yeast had the same effect, but killed bacterial cultures or watery extracts of yeast were ineffective. Bacillus coli was always effective when alive. The acidity and alkalinity of the different solutions were controlled. The author attributed the phenomena to the sense of smell of the larva and gave an exhibition of larvae feeding on stained bacteria (p. 178).

A rapid succession of different fungi occurs on manure (Gloyer and Fulton, '16, p. 6) and on other decaying substances together with an accompanying variety of odorous by-products. This succession determines the regular order in which decaying animal bodies become infested with insect scavengers. Mégnin ('85) and Hough ('97) have found that the order in which insects attack a decaying body is so constant that they have been able to develop a table giving the sequence of the different species. Thus

there are three stages of putrefaction and a final stage in which the dried tissues are consumed. The first stage of putrefaction is divided into two parts, viz., 'Body still fresh;' fauna consists of Musca domestica, Calliphora, etc.; 'Putrid odor develops;' fauna consists of Calliphora, Lucilia, Sarcophaga, etc. The workers of the second stage of putrefaction when butyric fermentation is taking place are Dermestes, Necrobia, Anthomyia, etc. The third stage, the stage of ammoniacal fermentation, is accompanied by infestation with such forms as Silpha, Necrophorus, Hister, Aphyra, Phora, and many Acarina. Finally the dried tissues are consumed by Aglossa, Tinea, Anthrenus, etc., and the bodies of these are destroyed Ptinus.

As this succession of species in the fauna of dead bodies holds fairly constant, it seems plausible that the odors produced are the determining factor and that the microörganisms producing the odors are of great importance to the insect as food and as solvent agents. Therefore we may conclude that the odors of fermentation and putrefaction are attractive to insects because they indicate a substratum made suitable for the insect by the abundance or the action of microörganisms.

MICROÖRGANISMS AS FOOD OF OTHER ANIMALS

The use of microphytes as food is not confined to insects and mites alone, but is quite common among Protozoa. The effect of pure culture of different species of bacteria on Paramecium has recently been described by Hargitt and Walter ('16). These authors were able to sterilize the animal by six successive washings in sterile water and then raised them on pure cultures of thirty different species of bacteria. They found that the bacteria from fresh were more favorable than those from older infusions.

It is probable that many of the Nematoda are also mycetophags. Anguillula aceti, the vinegar eel, which inhabits the 'mother of vinegar' and is also found in sour flour paste, and many of the 'parasitic' nematodes found in decaying plant tissue may be attracted at first by exposed soft tissue and later feed

upon the microörganisms in the decay with which they infest the plant.

Since Darwin's work ('81) it has been assumed that the earthworm finds its food in the humus of the soil it infests. Humus soil is notably rich in microörganisms, for these are the elaborators of humus from plant and animal remains, and it is possible that they are of food value to the worm.

Such structures as the endostyle and dorsal pharyngeal groove of Amphioxus and the Tunicates are probably for the purpose of entrapping microphytes of various kinds.

As pointed out by Osborne and Mendel ('14 b), microörganisms may also be of value to higher animals as elaborators of protein in the digestive tract from the non-protein substances ingested. This would be especially true in herbivorous animals, as Armsby ('11) has shown that non-protein substances are a source of protein in these animals, probably due to the formation of digestible bacterial protein in the digestive tract. The possibility that the flora of the digestive tract may modify the food elements supplied in a nutritional experiment is a drawback to the use of mammals in such experiments. An insect like Drosophila should be of value as material for such experiments because of the ease with which it is sterilized.

Many attempts have been made to rear mammals under sterile conditions, but most of these have failed so that it has been a great question whether or not it is possible for animals to live in the complete absence of microörganisms. As a large flora normally occurs in the digestive tract, it was necessary to sterilize the animal before it had taken food and to keep it in a sterile environment, therefore Pasteur ('85) suggested the use of hens, the eggs being well fitted for sterilization. Pasteur's suggestion was later carried out, but the first experiments were made by Nuttall and Thierfelder ('95–'96) on guinea-pigs, the young being removed aseptically from the mother by cesarian section. The animals were kept in a complicated aseptic environment and were fed upon food of animal origin. The animals gained 10 grams in one week (84 grams total weight) and appeared to be normal on the eighth day, when the experiment had to be discontinued.

In 1896 to 1897 the experiment was repeated, the gain in weight in eleven to thirteen days was almost normal, being 108 to 132 instead of 130 to 180 grams. In a third paper ('97) the authors experimented with the hen's egg and found that it was not sterile; they also summed up their previous work in the conclusion that animals just born do not grow well in the absence of microorganisms. Schottelius interprets the increase in the weight due of guinea-pigs in these experiments as due to the coagulation of caseinous material, from the milk, on the lining of the digestive tract. This author used hen's eggs as material to sterilize, but after a brilliant series of experiments ('99, '02, '08) has arrived at the conclusion that normal life without bacteria is impossible, as all the sterile individuals reared are retarded and stunted. Mme. Metchnikoff ('01) and Moro ('05) obtained similar results with tadpoles. In 1908 Tibbert, from theoretical considerations, came to the conclusion that higher organisms cannot live in the absence of microörganisms because each species of animal harbors definite numbers and species of bacteria. Metchnikoff, Weinberg, Pozerski, Distaso, and Berthelot ('09), on the other hand, reared the fruit bat Pteropus medius to normal size under practically aseptic conditions, and Cohendy ('12) kept chicks alive in anaseptic condition from twelve to forty days. Cohendy's conclusions are as follows:

> La vie sans microbe est possible pour un vertébré—le poulet—pourvu normalement d'une riche flore microbienne.
>
> Cette vie aseptique n'entraîne par elle-même aucune déchéance de l'organisme.

Kianigin ('17) has recently reopened this question by a review of all the literature. At first sight it appears incomprehensible that aseptic life should be so difficult when the greatest quantity of microörganisms is located in the non-digestive portions of the digestive tract. Metchnikoff ('09) points out, however, that the digestive powers of newly born are much weaker than those of older animals. The increasing number of cases of organisms which can be raised aseptically indicates that an aseptic existence may be possible in the majority of cases.

Nencki ('86) gave indirect evidence in this direction when he showed that the action of digestive ferments on food-stuffs makes them very quickly soluble and absorbable. The action of bacteria merely carries the decomposition to a lower level, yielding unassimilable aromatic acids, fatty acids, phenol, kresol, indol, skatol, carbon dioxide, methan, etc. The indications are, however, that microörganisms are of value as intestinal flora not because of their digestive, but because of their synthetic power.

MICROÖRGANISMS AS INTERNAL SYMBIONTS OF INSECTS

In addition to the cases of internal symbiosis of fungi with insects inhabiting dry wood, many Hemiptera and Blattidæ are also associated with microörganisms. These are usually bacteria or yeasts and infest the ovary. For example, the pseudovitellus of the aphid was long a puzzle to embryologists, but finally proved to be a granular body containing yeast cells which in the further development of the insect make complicated migrations and finally become lodged in certain fat-bodies which are termed mycetocytes or bacteriocytes, after which the infection of the ovary takes place. The evidence that a real symbiotic relationship exists in the Blattidæ and Hemiptera is given by Buchner and others as follows:

1. All eggs are infected.
2. The infection is not injurious to them.
3. Each species of insect is associated with a definite species of microörganism.
4. This association is very definite and almost a specific character.
5. The yeasts profit by the relationship in the protection which they receive from the host against the vicissitudes of the environment.
6. The yeasts multiply as the animal multiplies, always being present in constant amount even in such rapidly increasing forms as aphids and coccids.
7. The yeasts and bacteria are of value to the host as destroyers of waste products of metabolism, such as urates, according to

Sulc ('10b), and as absorbers of excess food materials such as sugar, according to Pierantoni ('10).

The movements and location of the symbionts has been studied by Buchner ('12) in a great work on the Blattidæ. Glasgow ('14) has also studied a case of symbiosis in which the microörganism, instead of being located in a fat-body (mycetocytes), as in the cockroaches, is retained in very highly developed gastric caeca of the plant bugs (Heteroptera). The function ascribed to the bacteria by Glasgow is the prevention of infection of the digestive tract by other bacteria. Dissected digestive tracts on bacterial media gave only pure growths of the associated microörganisms. Petri ('04, '05, '06) studied the similar case of Dacus oleae, which feeds on the olive, but ascribes to the bacillus alipolytic enzyme of assistance in the digestion of the food. Schaudinn ('04) finds that a fungus (Entomophthorineae) is transmitted through the egg of Culex and is always found in the diverticulae of the oesophagus of adult Anopheles and Culex. He has been able to rear the fungus in sugar solution and has demonstrated that CO_2 is formed in the imago from the sugars in the blood which it has sucked up. It is probable that the irritation caused by the bite of mosquitoes is largely due to enzymes secreted by these fungi.

In these cases the associated fungus may be a commensal, in its relation to the host, profiting by an oversupply of some food substance, as in the case of aphids, mosquitoes, etc., or may be of value as a chemical agent, as in the case of Dacus oleae, or may be of service in maintaining an unchanged digestive flora, as in the Heteroptera, as described by Glasgow. In general, however, the exact function of the microörganism to its host has not been thoroughly explained.

CONCLUSION

I have shown by experiments that Drosophila living in fermenting fruit are dependent for their food supply on the synthetic and absorptive powers of yeast cells. In a similar manner, my study of the relation of Musca domestica to manure, of Desmometopa to decaying meat, and of Sciara and Tyroglyphus to decaying

wood shows clearly that these Arthropods also feed on microörganisms. I have also endeavored to account for the origin and devolopment of this habit, to ascertain the probable extent of its occurrence, and to consider the known associations of animals with fungi in general. The experiments and considerations all tend to establish the principle that insects inhabiting fermenting and decaying substrata of low protein content, usually feed upon the microörganisms present and thus benefit by the power of the fungi to extract, adsorb, and synthesize many non-protein nitrogenous compounds.

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