Lipid Requirements of the Larva of the Housefly *Musca vicina* (Macq.) Reared under Non-aseptic Conditions

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(Received 26 January 1954)

Up to the present, no nutritional studies of the genus *Musca* have been made, despite the fact that *M. domestica* is extensively used for the laboratory testing of insecticides, and that in recent years, with the development of resistance to insecticides, the importance of physiological studies of the housefly has increased.

In general, the nutritional requirements of insects for lipids are mostly restricted to the unsaponifiable fraction (Scoggin & Tauber, 1950), although the saponifiable fraction (fatty acids) has been shown by Fraenkel & Blewett (1947) and by Pepper & Hastings (1943) to be related to normal growth, wing development, diapause and sterility.

The part played by the sterols in the nutritional requirements of several insects has been studied under non-sterile conditions; blowfly larvae by Hobson (1935) and *Dermestes*, *Tribolium*, *Lasioderma*, *Silvanus*, *Ptinus* and *Ephestia* by Fraenkel & Blewett (1943). As far as is known, bacteria neither synthesize nor require sterols (Deuel, 1951), although some yeasts contain phytosterol.

Our own experiments preliminary to the present work indicated that the bacterial flora of our laboratory-bred housefly failed to make good the nutritional deficiencies suffered by the larvae when reared on a fat-free diet. With this information, an investigation was undertaken into the lipid requirements of *M. vicina*. Some of the results of our work have already been reported briefly in a preliminary note (Bergmann & Levinson, 1954).

MATERIALS AND METHODS

A laboratory-bred strain of *M. vicina* provided the fly eggs and larvae used in the present experiments. The source of the stock bacterial flora and the method of its maintenance have already been described by Silverman & Silverman (1953).

For each test diet, 25 g. of a dry test medium were mixed with 25 ml. of sterile distilled water. The resulting 50 g. of wet medium were divided among seven test tubes (150 × 15 mm.), so that each tube contained approximately 7 g. of medium. Several hundred fly eggs, up to 12 hr. old, were washed in distilled water and added to a suspension

of stock bacterial flora in a Petri dish; the suspension contained between 1.5 and 2.5×10^8 bacteria/ml., of which 70% consisted of *Escherichia coli* and the remainder mainly of *Sarcinia*, *Lactobacillus* and *Bacillus subtilis*. By means of a pipette, 20–25 eggs in 1 ml. bacterial suspension were transferred to each of the test tubes containing wet medium, which were then incubated at 35°. The internal temperature of the test tube during the period of larval growth was 36°. To ensure that different diets received samples from the same mixture of eggs and bacteria, seven to ten diets were tested at the same time. One test tube out of the seven used for each diet was opened for examination on successive days until the time of pupation. At pupation time (usually the 5th to 7th day) the remaining tubes were opened and the pupae were pooled for weighing. Records were kept of the number and average weight of the larvae recovered daily. The mean weight value for each point on the curve was calculated from the collected average daily weights of larvae reared in replicate experiments. Each mean growth curve in Fig. 1 represents the growth of 1500 larvae. To allow for the differences in the age of the eggs, slight adjustments were made between the growth curves obtained from different series of the same test diet.

Control diet (no. 1). This consisted of wheat bran, obtained from the Golan Flour Mills, Tel Aviv. Before use, the bran was covered with pure ether and the ether was allowed to evaporate in a stream of air.

Basic lipid-free diet (no. 2). This diet was prepared by extracting 200 g. wheat bran with seven successive 1.5 l. portions of ether and then with one portion of 0.75 l. CHCl_3 . The extraction time with each portion of solvent was at least 24 hr. The CHCl_3 extract (concentrated to 20 ml.) was tested for sterols by the Liebermann-Burchardt reaction (Fieser & Fieser, 1949). In each sample, the extract proved to be sterol-free, thus indicating that the extraction with ether had been complete.

Basic lipid-free diet supplemented with total lipids (no. 3). The total lipids were recovered from the extracting solvents mentioned above and added to the basic lipid-free diet no. 2. The added lipids (4% of the total weight) were dissolved in ether, poured on to the dry media, and the ether, which was just sufficient to cover the media, was evaporated, leaving an evenly distributed residue.

Basic lipid-free diet supplemented with yeast extract (no. 4). This consisted of diet no. 2 plus 1% dried yeast extract. The yeast preparation, a water-extract of lysed yeast cells, was obtained from the Baltimore Biological Laboratories, U.S.A.; it was tested and found to be free from sterols.

Basic lipid-free diet supplemented with the saponifiable lipids (no. 5). This diet consisted of diet no. 2 plus 1% of the saponifiable wheat-bran lipid. The technique of Hilditch (1947) was followed in preparing the saponifiable fraction.

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Care was taken during the saponification and the subsequent distillation of the extracting solvent to prevent oxidation of the fatty acids by keeping the material in an atmosphere of CO_2 .

All the other diets were prepared from diets nos. 2 and 4 with supplements.

Diets nos. 1-8 were designed to test the nutritional value of the ether-soluble vitamins, and the saponifiable and unsaponifiable fractions of the lipids contained in the wheat-bran ether extract. Diets nos. 9-14 were prepared in an attempt to investigate the active constituent of the unsaponifiable lipids. The results are summarized in Fig. 1.

RESULTS

It was found that growth curves of replicate batches of larvae reared on the same diets showed variations of $\pm 10\%$ at each point on the curve, while pupation times differed by $\pm 5\%$. Larvae reared on different diets and whose resultant growth curves and pupation times differed from one another within these limits are represented by a single curve; e.g. in Fig. 1, curve *B* for diets nos. 3 and 8, curve *C* for diets nos. 9-13, curve *D* for diets nos. 2 and 5, and curve *E* for diets nos. 4 and 7.

Total lipids. A comparison of growth curves *A* and *D* in Fig. 1 shows the reduced growth rate and the lower maximum weight of larvae reared on a lipid-free diet. 3% of the larvae reared on diet no. 2 (curve *D*), pupated on the 7.5th day, 2 days after 90% of the larvae reared on the control diet no. 1 (curve *A*) had pupated. When the total lipids were replaced, as in diet no. 3, the resultant growth rate and maximum weight (curve *B*) were greater than those observed in the control larvae.

Ether-soluble vitamins. The effect on larval growth of the ether-soluble B vitamins is demonstrated by the growth curves resulting from diets no. 2 (curve *D*) and no. 4 (curve *E*). The yeast-

extract supplemented diet no. 4 supported growth of the larvae at a higher rate than the lipid-free diet no. 2. Although only 10% of diet no. 4 larvae pupated, pupation took place a full day before those reared on the lipid-free diet. The lower maximum weights of larvae reared on diet no. 4, as compared with those on diet no. 2 may be due to the earlier pupation time.

Saponifiable lipids. Larvae reared on diet no. 5 did not differ from those reared on the lipid-free diet (curve *D*). When larvae were reared on diet no. 7 (the lipid-free diet supplemented with yeast-extract and saponifiable lipids), the resulting growth was the same as that obtained on diet no. 4.

Unsaponifiable lipids. Curve *F* demonstrates the growth of larvae reared on diet no. 6. 50% of the larvae pupated on the 6th day. In the presence of yeast-extract (diet no. 8) the unsaponifiable fraction

Fig. 1. Mean growth curves and pupation times ($\downarrow$) of *M. vicina* larvae reared on various diets: *A*, diet no. 1; whole wheat bran. *B*, diets nos. 3 and 8; lipid-free wheat bran re-combined with the total lipid extracted, and lipid-free wheat bran vitamin enriched and supplemented with various sterols. *C*, diets nos. 9-13; lipid-free wheat bran, vitamin enriched and supplemented with various sterols. *D*, diets nos. 2 and 5; lipid-free wheat bran supplemented with saponifiable fat. *E*, diets nos. 4 and 7; lipid-free wheat bran vitamin enriched and supplemented with saponifiable fat. *F*, diet no. 6; lipid-free wheat bran supplemented with unsaponifiable fat. *G*, diet no. 14; lipid-free wheat bran vitamin enriched and supplemented with cholestan-3-one, and whole wheat bran supplemented with cholestan-3-one.

Table 1. Other diets

Diet no.	Basic diet no.	Supplements
6	2	Unsaponifiable wheat-bran lipids (0.17%)*
7	4	Saponifiable wheat-bran lipid (1%)
8	4	Unsaponifiable wheat-bran lipid (0.17%)
9	4	Wheat-bran sterol (0.17%)†
10	4	Sitosterol (0.17%)
11	4	Cholesterol (0.17%, m.p. 149°)
12	4	Cholesteryl acetate (0.17%, m.p. 114°)
13	4	Cholest-4-en-3-one (0.17%, m.p. 81°)
14	4	Cholestan-3-one (0.17%, m.p. 129°)

*Recovered from saponification carried out in preparation of diet no. 5.

† Prepared from the unsaponifiable fraction according to the digitonin precipitation method of Windaus (Abderhalden, 1925). The sterols were identified spectrographically (Fieser & Fieser, 1949) as consisting largely of sitosterol. A solution in cold CHCl_3 saturated with SbCl_5 was yellow, having an absorption max. at 500 μ . The infrared spectrum was also determined on 12 mg. of sterol in 1 ml. CS_2 , using a 1 mm. NaCl cell.

had a profound effect on larval growth. Diet no. 8, like diet no. 3 (curve *B*), produced larvae which were larger than those obtained on the control diet (curve *A*).

Sterols. When combined with yeast-extract, wheat-bran sterols (diet no. 9) sitosterol (diet no. 10), cholesterol (diet no. 11) cholesteryl acetate (diet no. 12) and cholest-4-en-3-one (diet no. 13) furnished a satisfactory medium for the larvae. Curve *C* is for diet no. 9; very similar curves were obtained with diets 10-13. Pupation took place on the 5th day, the earliest recorded pupation time. Cholestan-3-one, however (diet no. 4, curve *G*), appeared to inhibit growth and pupation, the larvae usually dying between the 6th and 7th day. The inhibiting activity of cholestan-3-one was found to be effective even in the presence of sitosterol.

Effect of temperature. Fig. 2 demonstrates the effect of temperature on the growth and pupation of *M. vicina* larvae. The larvae reared under the constant temperature of 36° grew faster and pupated 2 days before larvae reared in the same media, but subject to changing fermentation temperature which varied from room temperature on the first day to 42° on the 8th day. The growth represented by the variable temperature curve is typical of larval growth recorded from the usual laboratory housefly-breeding techniques which do not control the fermentation temperatures of the medium.

DISCUSSION

The general need of insects for a dietary lipid is confirmed for *Musca vicina*. Since by separating and then recombining the lipid and non-lipid

fractions of wheat bran as in diet no. 3, one is able to produce a medium which is more efficient than the whole wheat bran, it appears that the more lipid is available, the more effective is the diet.

It was not possible to prevent, during preparation of the lipid-free diet, extraction of some growth factors not usually considered as lipoidal substances. Certain of the B vitamins required by insects are soluble in fat solvents, e.g. thiamine, riboflavin, pyridoxine, pantothenic acid and *p*-aminobenzoic acid. These were removed to some extent along with the lipids by the extraction process. It appears from growth obtained on diet no. 4, that the role of these growth factors is insignificant when they are added in the absence of lipids.

It becomes clear from the results of diets nos. 5 and 7 that the saponifiable fraction of the wheat-bran lipids are not required by housefly larvae for growth and pupation. On the other hand, the unsaponifiable fraction is very active. The unsaponifiable fraction is unable, however, to act fully in the absence of the B vitamins. The effect of the unsaponifiable fraction could also be produced by its sterol component. The wheat-bran sterol, which is mainly sitosterol, is a relatively non-specific requirement since it, in turn, could be replaced by cholesterol, cholesteryl acetate and cholest-4-en-3-one. The corresponding saturated ketone, cholestan-3-one, appears to act as an antagonist. Fraenkel, Reid & Blewett (1941), van't Hoog (1935) and Hobson (1935), working with *Dermestes*, *Drosophila* and *Lucilia*, have also shown that a wide variety of sterols can satisfy the respective lipid requirements. It is therefore interesting to note that two closely related steroids, such as cholestan-3-one and cholest-4-en-3-one, have such markedly different effects. Whereas cholest-4-en-3-one is able to satisfy the sterol requirement of *Musca vicina*, it has proved to be inhibitory for larvae of *Aedes aegypti* (Golberg & De Meillon, 1947).

The steroids appear to act as both growth and pupation factors for *Musca vicina*. Both Gay (1938) and Golberg & De Meillon (1947), working with *Dermestes* and *Aedes* respectively, have shown that the effect of fat in the larval diet is manifest principally at the time of pupation and metamorphosis, which suggests that the sterol requirements are closely related to the moulting and pupation hormones demonstrated by Wigglesworth (1950) and Fraenkel (1935).

From diets which lacked a source of sterol, a number of flacciform larvae were usually recovered, which were unable to resist infection from pathogenic bacteria. Hobson (1935) also observed this susceptibility to infection, correlating the resistance of blowfly larvae to bacterial infection with the presence of cholesterol.

Fig. 2. Mean growth curves and pupation times (↓) recorded from *M. vicina* larvae reared in wheat bran: (A) at a constant temperature of 36°; (B) at an uncontrolled fermentation temperature varying from 30 to 42°.

SUMMARY

1. The lipid requirements of the larvae of the housefly, *Musca vicina* have been studied. The only lipid required is contained in the sterol fraction of the wheat-bran medium.

2. This sterol was identified as sitosterol; however, cholesterol, cholesteryl acetate and cholest-4-en-3-one were found to produce the same biological activity as sitosterol. Cholestan-3-one, on the other hand, does not replace cholesterol; moreover, it is an antagonist of the normal 'active' steroids, inhibiting growth and preventing pupation.

3. The role of the sterol as an agent in larval resistance to bacterial infection, as a pupation factor, and as a promoter of growth is discussed.

4. The experimental rearing method demonstrates that a constant temperature of the medium can accelerate growth and pupation when compared with the usual breeding methods in which fermentation temperatures of the medium are uncontrolled.

Acknowledgement and thanks are made to Professor Ernst D. Bergmann whose aid and counsel made this work possible, and to Miss Judith Byk for technical assistance.

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Studies on the Lipids of *Musca vicina* (Macq.) during Growth and Metamorphosis

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(Received 26 January 1954)

It has been shown in a previous paper (Silverman & Levinson, 1954) that *Musca vicina* requires a sterol as its sole essential 'lipid'; it is apparently unable to synthesize sterols. It was found that larvae could develop to the third stage when reared on a sterol-free diet, but were well below the average normal weight and failed to pupate. In order to determine the relation of sterols and other lipids to the developmental periods of the normal housefly larva, a systematic analysis of lipid at various stages of development was undertaken. It was felt that such an analysis might yield useful information in regard to *M. vicina*'s ability to utilize and synthesize lipids. Similar studies by Frew (1929), Evans (1932), Yüill & Craig (1937) and Haub & Hitchcock (1941) on blowfly larvae have been mainly concerned with changes in the saponifiable fraction of larval fat.

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Evans (1934), however, recorded observations on the unsaponifiable fat of *Tenebrio molitor* during metamorphosis and found that it remained constant throughout.

MATERIALS AND METHODS

Housefly breeding. The technique for stabilizing the microflora and density of larvae breeding in the medium has been described in full in a previous paper (Silverman & Silverman, 1953). A short summary of the technique is given here.

The housefly larvae were reared in round glass containers, 20 cm. in height and 15 cm. in diameter. The breeding media consisted of the following thoroughly mixed ingredients: 300 g. of wheat bran, 40 g. straw and 300 ml. water. The jars were covered with a double layer of cloth towelling which was held in place by a metal rim. They were sterilized by autoclave and then allowed to cool to room temperature before seeding with eggs. Each jar was seeded with 1000–1250 eggs in 20 ml. water suspension of