BIONOMIC AND ENZYMATIC STUDIES OF THE HOUSEFLY IN RELATION TO DISEASE TRANSMISSION

by

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GENERAL INTRODUCTION

The housefly has probably been implicated as a vector of more diseases than any other species of animal. Due to the varied means of dissemination of the enteric diseases, and the accidental rather than biological association of the fly, evidence establishing its guilt has, of necessity, been indirect. The mass of evidence has, nonetheless, been imposing, and there is little doubt that the housefly is potentially important in the transmission of bacillary dysentery, typhoid, cholera, and possibly the viruses of poliomyelitis and Coxsackie. Despite its importance from a Public Health and nuisance point of view, some gaps remain concerning our knowledge of fly biology; for example, it is not known how the housefly overwinters in Kansas. The ecological aspect of vector biology is fairly well studied, however, compared with the scant data on the host itself as an ecological niche for the parasite.

The emphasis in studies of human diseases has been on the pathological sequelae resulting from an invasion of the host by an organism. This undoubtedly has its roots in the notion that disease itself is an unnatural phenomenon. Theobold Smith dispelled this

notion, in his fundamental series of talks on parasitism, when he set forth the doctrine that disease is an expression of a biological interaction of two organisms and is essentially an overlapping in their life cycles. This viewpoint encourages the study of host biology in relation to the requirements of the invading parasite. Disease and ultimate death of the host are indicative of a poor adjustment between the host and parasite. Before the advent of modern medicine and antibiotic therapy, a process of selection was undoubtedly going on, in which less virulent organisms and more resistant hosts were favored.

Infection with an organism is not synonymous with disease. It is increasingly appreciated for many diseases that the majority of infections are without disease symptoms; only in a small percentage of cases, and for usually unknown reasons, is the host-parasite balance upset resulting in disease symptoms. In diseases such as dysentery, typhoid and poliomyelitis, where infection without disease is not unusual, man shares the role of vector, or more accurately carrier, with the fly. The difference is that flies, so far as we know, never succumb to the agents they habitually carry to man. If conditions in the vector, fly or man, are favorable

and multiplication of the parasite occurs, then the vector efficiency is increased. It is only necessary, however, for the agent to persist long enough to be passed on to a susceptible host.

Practically nothing is known concerning the natural history of infection in the fly. Does the organism remain in the gut of the fly, and if so, where is it most concentrated? Does multiplication take place, and if so, what is the optimum temperature and does the host's diet have any effect? What are some of the fly's defenses? Is there a normal phage as well as bacterial flora encountered in the gut by an invading organism? Does the midgut with its low pH act as a bactericidal chamber, much the same as our own stomachs; and if once established, are antibodies produced against the parasite?

These are some of the problems which need to be studied in order to achieve a better understanding of the biological interrelationship between the housefly and some of the disease organisms which it disseminates. The following group of studies is merely a small step in that direction.

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PART I. SURVIVAL OF ENTERIC PATHOGENS THROUGH PUPATION

Introduction

Faichnie (1909) appears to have been one of the few early workers to demonstrate the survival of an enteric pathogen through pupation in the housefly. His results are not entirely convincing, however, because of the possibility of re-infection of

emerging adults from unsterilized pupa cases. The consensus among early workers was that enteric pathogens, like the Salmonellae of typhoid and food poisoning, and the Shigellae of dysentery, could not survive pupation in the housefly or other muscoid flies, under normal conditions (Ledingham, 1911; Nicoll, 1912; Graham-Smith, 1913; Krontowski, 1913; Tebbut, 1913; Wollman, 1921). More recently, Ostrolenk and Welch (1942) succeeded in demonstrating the survival of Salmonella enteritidis through pupation in the housefly. It is assumed from their work that pools of larvae or adults were tested rather than individuals. Since one infected individual in a pool of 100 or more flies could render the pool positive, there is no way of knowing what percentage of adults remained infected after pupation. Gerberich (1952) found about 41 per cent of emerging flies still infected with Salmonella pullorum.

In the light of these recent findings and the introduction of highly selective media, unavailable to the early workers, it was decided to repeat some of the previous experiments.

Materials and Methods

A strain of Musca domestica L. collected in Lawrence, Kansas, was used for this work. Eggs were placed in 125 cc Ehrlenmeyer flasks containing cellu-

cotton moistened with a mixture of powdered milk and honey. The initial pH of this medium is about 7.1 and increases to about 8.2 as the larvae mature. At 36° C, the cycle from egg to adult is about 11 days. The following microorganisms were used in this study: Salmonella typhosa (Stephens strain) isolated as a Vi type from an active case and maintained on veal infusion agar for about one year prior to this work, S. paratyphi B, and Shigella flexneri 6, the last two generously forwarded by Dr. P. R. Edwards of the Communicable Disease Center, Public Health Service.

When the larvae entered the third instar, 5 cc of a 24 hour broth culture of the test organism was introduced to the flask, and 24 hours later, maggots were removed for infectivity tests, conducted as follows: 8 to 10 maggots were rinsed several times in sterile water and then immersed, with frequent agitation, in a 0.2 per cent Roccal solution for 1 hour at room temperature. They were then rinsed several times in sterile water and individually passed through small tubes of tetrathionate broth, as a test for the disinfection of all organisms able to grow on the selective media employed. After 18 hours incubation in this enrichment medium, bismuth-sulfite agar and SS agar were streaked. Generally, no colonies were found on

either medium. Though complete disinfection is more desirable, it was felt that a disinfection procedure which completely destroyed the test organism would be sufficient for the needs of the work. Following disinfection, the maggots were ground with the aid of a sterile glass rod in individual tubes of tetrathionate broth and the same procedure followed as above. Typical colonies were picked to Kligler's iron agar slants after 24 to 48 hours. In this medium, Salmonella typhosa produces an alkaline slant, acid butt, H_2S along the stab, and no gas; S. paratyphi B produces an alkaline slant, acid butt; somewhat more H_2S, and gas; while Shigella flexneri 6 produces an alkaline slant, acid butt, no H_2S and no gas. Positive reactions in this medium were confirmed by agglutination tests with typing serum. For S. typhosa, both Vi and O sera were used, while for S. paratyphi B and Sh. flexneri 6 group specific sera were employed which had been obtained from Dr. Edwards.

For the work with S. typhosa, selenite broth was used as the initial enrichment medium, but tetrathionate broth proved to be more convenient as regards incubation period and was used for the other two organisms.

As reported by previous workers, it was found difficult to completely disinfect pupae, presumably because the internal surface of the spiracles resist

wetting with the disinfectant and thus retain viable microorganisms. Various disinfecting procedures were tried, including the following: 1. 30 minutes in 0.2 per cent Roccal in a 37° C water bath; 2. 20 minutes in 0.5 per cent Roccal at room temperature; 3. 4 minutes in 70 per cent alcohol containing 1:500 $HgCl_2$; 4. immersing in 70 per cent alcohol and igniting same. Where disinfection proved most effective, as with # 3 above, eclosion was poor, so that relatively large numbers of pupae needed to be processed to obtain a few adults. Pupae were shaken in broth tubes for 25 minutes before being transferred to tubes of moist, sterile sand which were stoppered with a sterile cork. If the broth tubes showed growth, the pupae were discarded. Young imagoes were ground in 2 or 3 cc of tetrathionate broth and processed as for larvae described above.

Results

The results of these experiments are shown in tables 1, 2 and 3.

Discussion

The data appear to indicate differences in the abilities of S. paratyphi B on one hand, and S. typhosa and Sh. flexneri 6 on the other hand, to survive pupation and appear in emerging adults. Early workers

generally assumed their larvae were infected without testing them. In this study, whenever the organism could be recovered from larvae, it was usually recoverable from adults, but in lesser numbers. When infection could not be demonstrated in larvae, it generally could not be shown in adults either. It is possible then, that loss of infectivity in adults is not due entirely to phagocytosis or other events during pupation which may destroy bacteria, but also to as yet unexplained factors in the maggot's gut which may help to destroy large numbers of the test organism before pupation. Melnick and Penner (1952) found that early third instar larvae of Phaenicia sericata (Meig.) infected with poliomyelitis or Coxsackie virus retained only minimal amounts of the virus 1 and 3 days after feeding and none of the adults was infected. Besides a possible antibiotic effect of the normal flora of the fly gut, other factors like pH, enzyme activity and bacteriophage may serve to create an unfavorable milieu for the survival of certain enteric pathogens. The relation of these factors to the differential survival of organisms, such as the ones used in this study, needs more elucidation.

Hawley, et al, (1951) demonstrated multiplication of Sh. dysenteriae and S. paratyphi B (=

schotmülleri) fed to adult houseflies, provided the infective dose was above 1,000 organisms. It is possible that a process of selection is operative here, leading to the survival of a strain of organisms better adapted to conditions in the fly's gut. If this is so, continued re-passage of the organism through houseflies should result in a significant reduction in the size of the infective dose.

Ostolenk and Welch (1942) have shown that adults infected with S. paratyphi A (= enteritidis) remain infected for life (about 28 days). Faichnie (1909) considers the gut of the fly and not its exterior to be the greater source of infection, and Wollman (1921) found that adults could re-infect themselves from the exterior of the pupa cases but would remain infected for only a few days. Graham-Smith (1913) found that Serratia marsescens may survive for at least 18 hours on the wings and legs of the fly infected during feeding, but that the intestine may remain infected up to 17 days. Thus, for organisms like S. paratyphi A and B, capable of surviving the larval and pupal stages, infection of adults may occur in the breeding as well as feeding grounds; whereas organisms like S. typhosa and Sh. flexneri 6 which do not survive from the larva to the adult, must re-infect the imago and such re-infection may be less frequent and more transitory in nature.

TABLE I. SURVIVAL OF S. PARATYPHI B THROUGH PUPATION

Experiment	Larvae infected	Per cent	Adults infected	Per cent
1	0/25	00.0	10/14	71.5
2	10/20	50.0	2/15	13.3
3	0/14	00.0	0/58	00.0
4	5/7	71.0	5/18	27.7
5	0/7	00.0	0/6	00.0
6	7/7	100	2/3	66.6

TABLE II. SURVIVAL OF S. TYPHOSA THROUGH PUPATION

Experiment	Larvae infected	Per cent	Adults infected	Per cent
1	1/8	12.5	0/19	00.0
2	1/28	3.6	0/13	00.0
3	0/28	00.0	0/4	00.0
4	0/28	00.0	0/15	00.0
5	0/28	00.0	0/7	00.0

TABLE III. SURVIVAL OF SH. FLEXNERI 6 THROUGH PUPATION

Experiment	Larvae infected	Per cent	Adults infected	Per cent
1	0/6	00.0	0/3	00.0
2	0/7	00.0	0/3	00.0

PART II. A NEW METHOD FOR THE STERILE CULTURE OF LARVAE

During the past decade, larvae of the housefly have been reared routinely in a medium containing two parts of wheat bran and one part of alfalfa meal moistened with a yeast suspension and a solution of malt extract. The recent appearance of a standard mixture of wheat bran (33.3 per cent), alfalfa meal (26.7 per cent), and brewers grain (40.0 per cent), called CSMA (formerly NAIDM), has further simplified the rearing of this species of fly. This study reports a modification of the CSMA medium which makes the rearing of maggots still simpler, while reducing the cost. This medium has also been successfully used in the sterile culture of larvae.

We have reared larvae routinely for the past

year in CSMA moistened solely with tap water. Approximately 400 cc of CSMA in quart canning jars are thoroughly mixed with 300 cc of tap water. Care is taken to stir the entire contents of the jar, leaving the medium loose and freely accessible to the growing larvae. Eggs are then introduced and the jar is placed in a 36° C incubator. Too few larvae mature if mold is abundant, while excessive crowding results in a " boiling over " of half grown larvae. It is interesting to note that temperatures in the interior of the larval medium rise to 41° C or 42° C during the period of active growth. When pupation begins, a screen cap is screwed on the jar and with the appearance of adults, the jar is placed in a cage.

Seven strains of flies were used, including some resistant to DDT. Two different lots of CSMA were employed. The developmental period was about 9 days, 4 days for the larvae and 5 for the pupae. Adults were of normal size, with a preoviposition period varying between 4.5 and 6 days, and laid fertile eggs.

During the course of studies on proteolytic enzymes in the housefly, it was found necessary to rear larvae under sterile conditions. The following method was used: Several 125 cc Ehrlenmeyer flasks, containing about 60 cc of CSMA moistened with 50 cc of tap

water were plugged and incubated at 36° C for 24 hours. Ten cc of water were then added to each flask and the flasks autoclaved for 2 hours at 118° C. Eggs were sterilized by Glaser's method (1938) using White's solution, and about 50 eggs introduced into each flask. Sterility tests were run for eggs and medium at this time, using thioglycollate broth which was incubated at 36° C and held for 48 hours. Aluminum foil was used to cap the cotton plugs of each flask which were then incubated at 36° C. The control consisted of the unautoclaved medium with treated eggs in a flask similarly plugged.

At the end of 3 days, test maggots were in the second instar and from 3 to 4.5 mm. in length, while the controls were mature third instars, about 11 mm. long. The latter pupated on the fourth day and the test larvae pupated on the sixth day. Maggots in 3 of 5 flasks died as mature second instars without further development; those in another flask died suddenly in an unaccountable manner as almost mature third instars; while those in the last flask pupated in the cotton plug giving rise to undersized pupae. Fully formed flies began appearing 6 days after pupation so that the minimum developmental period was 12 days. Additional sterility tests on the fifth and seventh days showed the maggots and medium to be sterile.

One flask, in which the maggots had grown as rapidly as the controls, proved contaminated.

These experiments were then repeated using larval media that had been incubated for 2 days at 36° C, and another batch that had not been incubated at all, but was autoclaved as soon as it was prepared. Table 4 summarizes the results of these experiments. With a 2 day incubation, larvae grew as rapidly as the controls and pupated in slightly less than 4 days. These pupae were of normal size. The larvae in the unincubated medium grew slightly during the first several hours after their emergence. Thereafter, growth ceased and they continued to wander about in the flasks until they died, 3 to 7 days after hatching, still in the first instar.

Presumably microorganisms and/or vitamins and other accessory food substances produced by the microorganisms during the incubation period and not destroyed by autoclaving are responsible for these results.

This simple method for rearing housefly larvae aseptically is not a substitute for a defined synthetic medium where the effect of known constituents may be significant. Where this is not important, however, the easy preparation and low cost of this medium recommend its use in more controlled studies of the housefly. For example, it is desirable in the study of maggot

enzymes to vitiate any possible effect due to enzymes produced by the normal flora of the gut. This medium also provides the means whereby investigations can be undertaken to determine what effect, if any, the presence or absence of the normal gut flora have on the survival of certain enteric pathogens. From an ecological and nutritional viewpoint, it would be of interest

TABLE IV. INCUBATION OF CSMA AND ITS EFFECT ON LARVAL GROWTH

Incubation period(hours)	Number of flasks with pupae
0	0/6[1]
24	1/6
48	5/5

to determine the specific microorganisms and their products which control the growth of larvae in this medium. Vitamin B_{12} produced by Lactobacilli is known to exert a significant effect.

1. Only 4 maggots reached the second instar, and 2 of these emerged as bacteria-free adults 13 days after hatching.

PART III. STUDIES OF PROTEOLYTIC ENZYMES

Introduction

Most workers have considered pepsin to be absent from insects (Chauvin, 1949; Wigglesworth, 1953) and indeed from all invertebrates (Florkin, 1949; Prosser, et al, 1950). Day and Waterhouse (1952) make no mention of this enzyme's occurrence in their review of insect digestion. Published observations of peptic activity in insects do exist but are preliminary in scope and generally inconclusive (Pavlovsky, et al, 1922; Brown, 1928; Mackerras, et al, 1933; Tomita, et al, 1936). Some make no reference to pH in their work, others refer only to a " distinctly acid " solution, while none recognize the possibility that some tryptic activity may remain at low pH levels. It is, therefore, of some interest for insect and comparative physiology that proteolytic activities at low pH levels have been found in the larval stages of the housefly. The high degree of proteolysis at pH 1.5 to 3.5 is strongly suggestive of the presence of pepsin or a pepsin-like enzyme[2], in at least the larvae and possibly in other stages as well.

2. The terms 'pepsin' and 'trypsin' are used throughout this paper for convenience, though the designations, 'pepsin-like' and 'trypsin-like', reflect more accurately the state of our knowledge concerning these enzymes in the housefly.

Materials and Methods

The same strain of housefly used in the previous studies was used for this work. The test sample (eggs, larvae, pupae, or adults) was rinsed several times in tap water and then in distilled water; all stages except eggs and early first instars were then dried on paper towelling, counted, weighed, and finally ground in cold Ringer's solution in a Teflon-pyrex tissue grinder immersed in an ice bath. The enzymatic studies described in this paper were made on these homogenates, unless otherwise indicated. While such homogenates may be termed 'unphysiological' they are of considerable value in enzyme research for revealing the existence of many enzymes which otherwise may go undetected. The use of homogenates, or breis, does not pretend to reveal the sequence of *in vivo* metabolic events; it does serve as a tool for individual enzyme study.

Egg quantity was computed on the basis that 0.1 cc of eggs equals about 500 and each egg weighs 0.0633 mgms. Early first instars were assumed to have about the same volume as the eggs, and the weight of one maggot equalled 0.0675 mgms. Breis obtained from third instar larvae, pupae and adults were filtered through glass wool; and aliquots of 1 cc were transferred with a tuberculin syringe to conventional Warburg manometric flasks. Three buffer systems were

used for the range pH 1 to 12: HCl-KCl (pH 1 to 2); phosphate-citrate (pH 2.5 to 8.5, 0.1 M); and phosphoric acid-acetic acid-NaOH (pH 9 to 12). Two cc of buffer, of appropriate pH, were added to the extract, and the substrate, 0.5 cc of a 1 per cent solution of the described protein, was added to the sidearm of the vessel. The flasks were secured to the manometers, placed in a 37° C water bath and shaken for 5 minutes before tilting the substrate into the buffered brei for a 2-hour incubation period with constant shaking. (Determinations of pH for these solutions after the 2-hour run showed increases of not more than 0.2 of a unit). The contents of each flask were then emptied into a tube containing 0.5 cc of a 29 per cent solution of trichloroacetic acid, the flasks rinsed twice with distilled water and the final volume adjusted to 5 cc with distilled water. After 30 minutes in the cold room, the tubes were centrifuged and 1 to 3 cc of the supernatant reacted in a spectrophotometer tube with 8 cc of 0.5 M NaOH, 1 cc 0.0025 M $CuSO_4$, and 3 cc Folin-Ciocalteau reagent (Herriott, 1941). The intensity of the resultant color indicates the number of phenolic groups present and is a measure of the degradation of the original substrate. Controls consisted of the following: 1. an aliquot of the homogenate in distilled water in lieu of substrate, regular incubation,

to neutralize the effect of non-specific autolysis (homogenate in buffered solutions at various pHs yielded no significant difference in amount of autolysis); 2. substrate in distilled water, and no homogenate, regular incubation; 3. homogenate and substrate at zero time. The first control always had more activity than the latter two. Colorimetric determinations were made with a Coleman Universal Spectrophotometer at 540 λ and were in optical density (O.D.) units. The optical density is directly proportional to the amount of digestion.

Known amounts of tyrosine (9.8 to 39.2 mgms. per 100 cc) were reacted with the colorimetric reagent and the resultant O.D. values were plotted against concentration of tyrosine. By means of the standard curve thus obtained, the O.D.s of the experimental samples were converted to tyrosine equivalents. A standard curve was plotted for each new batch of Folin-Ciocalteau reagent. Aliquots of brei were assayed for total nitrogen by means of the micro-Kjeldahl technique. Activity units (A.U.) were then computed as follows:

$$A.U. = \frac{(V_h)\ (V_m)\ (T.E.)}{(V_s)\ (W_s)\ (W_n)}$$

where,

V_h = initial volume of homogenate

V_m = final dilution volume of reaction mixture

T.E. = tyrosine equivalent

V_s = volume of supernate from V_m reacted with Folin-Ciocalteau reagent

W_s = mgms. of original sample

W_n = mgms. of nitrogen in homogenate

Results

With sodium caseinate as substrate, all the larval stages exhibited strong proteolytic activity with a pH optimum at about 2.5. Eggs, pupae and adults, on the other hand, had singularly consistent peaks at 3.0. A further difference separates the enzymes at the low pHs, and this is the per cent of the activity at the optimum pH remaining at pH 1.5. In larvae, activity at this pH is 61 to 96 per cent of the peak value; in eggs, it is 0 per cent; in pupae, it ranges from 0 to 22 per cent with no apparent correlation with age; and in newly emerged adults it ranges from 7 to 20 per cent. Breis of second instars incubated with bovine albumin (Armour's) or hemoglobin (Nutritional Biochemicals) showed an optimum pH of 2, while eggs, pupae and adults maintained an optimum of 3.0 on bovine albumin. With sodium caseinate as the substrate, all stages but the eggs had a variable pH optimum for trypsin ranging from 8 to 10.5, but a peak between 8 and 9 was most repeated. (Powning, et al, (1951) report an optimum for trypsin at pH 8.5, using dissected midguts, pre-

sumably of third instars, on a gelatin substrate.)

Eggs had no tryptic activity, with either sodium caseinate or bovine albumin, while the trypsin of other stages was unable to cleave hemoglobin or bovine albumin. Table 5 summarizes pH optima for all stages, and figure 1 shows the pH activity curves.

Discussion

The enzymes found in the present studies on the housefly have been designated as pepsin, cathepsin and trypsin solely on the basis of the pH range for proteolysis. It is entirely possible that such enzymes may not be the same as the classic vertebrate pepsin or trypsin. The identity of these proteolytic enzymes can only be definitively established by appropriate studies of the purified material. Until such work is done, the author feels justified in referring to the enzymes as pepsin, or pepsin-like, trypsin and trypsin-like and catheptic on the basis of their pH requirements. It is well known that optimum pH for pepsin and trypsin activity will vary, depending on the nature and isoelectric point of the substrate, and the ionic strength of the buffer used.

Egg - Eggs tested on casein had two optima, at pHs of 3 and 5, diminishing to zero at neutrality. In 7 tests using 500 to 3,000 eggs per test there was

no digestion at pH 1.5. In sampling large numbers of eggs, the factor of age must be considered. This difficulty is not easily circumvented, for even eggs from the same egg batch differ in development. The question arises whether the older eggs in a sample, with distinctly visible maggots within them, tend to bias the results toward the activity curve characteristic of the early first instar. The effect of older eggs, if any, would be to lower the pH optimum and increase the activity at pH 1.5. The data do not indicate any such effect. There is a consistent optimum at pH 3 and zero activity at pH 1.5. No digestion was recorded in the alkaline range and it is assumed, on the basis of their pH requirements, that both proteinases in eggs are catheptic in nature.

First instar - A pH optimum at 2.5 is most frequently observed for this stage, while 61 to 77 per cent of peak activity remains at pH 1.5.

Second instar - The pH optimum for casein hydrolysis is the same as for the first instar; proteolysis of hemoglobin and bovine albumin are optimal at pH 2. These latter substrates were not hydrolyzed in the alkaline range. At a pH of 5, proteolytic activity is still 11 to 16 per cent of its peak activity on hemoglobin and 0 to 6 per cent of the maximum on albumin; no activity on either substrate remains at pH 6. Peptic activity at pH 1.5 is 82 to 96 per cent of the peak value.

Third instar - A phenomenon of trypsin suppression encountered in this and the following stages is discussed below. Where they were about equal in the previous stage, the relative activities of pepsin and trypsin now average about 9 to 1. This stage also varies most with respect to its optimum for pepsin but averages about 2.5. This was also the peak obtained with dissected guts. Optima at 3 may be due to selection of pre-pupae rather than the actively feeding larvae. Peptic activity at pH 1.5 is 62 to 74 per cent of the peak value.

Pupa - White, red and black pupae were tested separately on casein and albumin, and had consistent peaks at pH 3 and from 0 to 22 per cent activity at pH 1.5. If this is a catheptic enzyme, it differs from the ones in eggs in having greater activity below pH 3 and in falling off more sharply above it. The increase of trypsin in older pupae is discussed below.

Adult - Adults were sexed and separately tested as unfed, newly emerged, and fed, three-day-old flies, with no significant differences in enzymes characteristics. The relative activities of pepsin and trypsin are similar to that observed in third instar larvae.

Dissections of third instars in cold Ringer's solution demonstrated slight, if any, proteinase activity in the cuticle and attached tissues, in the fatty tissue, or in the salivary glands.

Guts of third instar maggots exhibited the usual degree of activity, with one interesting modification. Breis of dissected guts had considerably more tryptic activity than those of whole maggots. An inhibiting substance may be present in the homogenates of whole maggots and the following figures illustrate the range of this inhibition. The data express the per centage of tryptic activity in relation to peptic activity, derived from optical densities, using equal numbers of maggots of the same age and size $\left(\frac{\text{Trypsin activity}}{\text{Pepsin activity}} \times 100\right)$.

Experiment	Entire maggot	Gut
1	40%	100%
2	1%	50%

Homogenates of entire white pupae and adults also had slight tryptic activity. Seven separate runs for fed and unfed adults gave ratios ranging from 20 per cent to 3 per cent, while a single run with white pupae yielded 17 per cent. Older pupae show a progressive increase in tryptic activity; 48 hour pupae (at 25° C) showed a per centage of 40, and 96 hour pupae, a per centage of 85. Strangely enough, flies no older than 30 minutes, with wings still unexpanded, had ratios which reverted to 5 and 20 per cent, in 2 samples tested. It is considered unlikely that this

phenomenon is due to technique because of the uniformity of procedure for a fairly large number of experiments sometimes involving various stages within the same experiment. Since there is no apparent trypsin inhibition in second instars, extracts of second and third instars were mixed in various proportions. Although a reading as low as -24 per cent of the theoretical value was obtained in one case, others ranged around -6 per cent, while some were as high as 11 per cent. The readings do not indicate any marked suppression of the tryptic activity of second instars, under the conditions of the experiment. The inhibiting substance, if there be such, first manifests itself in the third instar, diminishes during pupation, rises rapidly at eclosion and continues to exert its influence in both sexes of the adult.

To preclude the effect of any enzyme activity due to microorganisms, maggots were reared aseptically, as described in Part II, and early third instars were ground and tested as usual with no significant differences in peptic and tryptic optima from those already described (table 5).

To further characterize the tryptic and peptic enzymes, a series of experiments were performed to determine temperature optima, using second instar larvae. Each experiment included duplicates at pH 2.5 and pH 9, run at three different temperatures. Employing the technique outlined earlier, we were limited by the

number of available Warburg water baths and, consequently, a clear-cut optimum was obtained for neither enzyme. The data indicate broad activity for trypsin between 34° C and 50° C, while pepsin, in most cases, has an optimum around 37° C. Both enzymes are more active at 37° C than at 25° C.

No peptic digestion occurs at 55° C or above, while the trypsin, at 55° C, still retains 90 per cent of its activity recorded at 37°C; at 60° C, 60 per cent; and at 70° C, about 8 per cent. Working with dissected midguts of third instars, and using a gelatin substrate at pH 8, Powning, et al, (1951) found considerably greater thermal inactivation. Fifteen minutes' exposure to 60° C destroyed half the tryptic activity, while at 70° C, less than 1 minute was necessary. One obvious weakness in the both approaches is the failure to adjust the experimental incubation period to approximate the duration of time that the substrate is exposed to the enzyme in the gut of the maggot. Temperature optima of in vitro systems are not necessarily the same as those obtained in vivo. In vivo, enzymes operating at lower temperatures may digest more total protein than at higher temperatures, for an equivalent extent of time. The above figures should be studied in this context.

Figure 2 shows an apparent though not neces-

sarily actual decline in both peptic and tryptic activity as the larva matures. It is reasonable to assume that with larval growth, the percentage of total nitrogen in enzymatic form decreases as the amount of nitrogen converted to cuticle, fat tissue, muscle and histoblasts, etc., increases. Pupation represents a stadium of greater stability for pepsin, but tryptic activity is on the increase during this time. It should be emphasized in a discussion of these figures that they are significant only in the broad ranges that they indicate and not as precise numerals.

On the basis of reported work, endopeptidases generally seem to occur singly in insects. Duspiva (1939), however, has reported catheptic as well as tryptic activity in the gut of Dytiscus. Cathepsin is itself an infrequent find in insects. Ballentine (1940) has also reported two proteinases from a dragon-fly nymph, one a typical trypsin and the other with a pH optimum around 5.0 to 5.5, which may be a cathepsin. The housefly has two distinct proteinases functioning in every active stage of its life cycle. Kowalewsky (1889) demonstrated that the midgut of the housefly larva is alkaline except for the middle region which is strongly acid. This suggests the regions in the midgut of the housefly where peptic and tryptic

digestion occur, leaving unexplained, however, a similar situation in the gut of blowflies where pepsin may not be present (Hobson, 1931).

Differences in food preferences exist within a species. For example, the Australian Phaenicia cuprina (Wied.) (= pallescens) causes myiasis in sheep, whereas the North American variety is a free living saprophage. Differences in food preferences in what is presumably the same species indicate as great a need for sampling widely dispersed populations of a species for physiological studies as for taxonomic ones. The same consideration applies to this study of the housefly, and it is well to call attention to this limitation in the present work. Enzyme studies might profitably be employed toward a clarification of the systematics of Musca species, as high-lighted by renewed investigations in recent years (Holway, et al, 1951; Sabrosky, 1952; Peffly, 1952; Sacca, 1953). It would be of some interest to learn whether the entire genus follows the same enzyme picture elucidated for M. domestica or if differences do occur which can be correlated with morphological, bionomic and genetic data.

TABLE V. pH OPTIMA FOR PROTEOLYTIC ENZYMES IN ALL STAGES*

The pH showing maximum activity is indicated for each experiment performed

Stage \ pH	1.5	2.0	2.5	3.0	3.5	8.0	9.0	10.5
Egg				5C 1A	1A			
First instar		1C	4C			3C	2C	
Second instar		1C 2A 2H	3C				2C	1C
Third instar	1C	1C	5C[3,4]	5C		3C[4,5]	2C	1C[5]
Pupa				3C 1A		3C	1C	
Adult				5C 1A		4C		

* Numbers indicate number of experiments on sodium caseinate (C), bovine albumin (A), and hemoglobin (H).

3 Two with dissected guts.

4 One with dissected guts.

5 One with sterile third instars.

PART IV. STUDIES ON FECUNDITY AND COLD SURVIVAL

Introduction

This study concerns the effect of isolation on the reproductive activity of houseflies, and also contains observations on cold survival of maggots and adults, and describes what is presumably a mutation in the mouthparts of larvae. The former study was undertaken, in part, to answer the need of the housefly resistance project in this Department for a homozygous strain of flies - best accomplished through consecutive brother-sister matings over several generations. Though no difficulty was encountered in obtaining seven successive generations of normally ovipositing flies, many early workers reported poor success with single pairs, so it was decided to study the problem further.

Hutchison (1916), using single pairs of flies, obtained egg deposition in only 7 out of 30 pairs, with a maximum of 110 eggs in 2 batches. He attributes this inhibition of oviposition to a 'psychological factor'. These observations were later confirmed by Glaser (1923). Roubaud (1922) found that isolated females in small cages laid fewer eggs and died in 10 days or less; he cites Awati (1920) who studied Musca nebulo Fabr. and M. angustifrons Thoms. and reported similar results.

Dunn (1923), Feldman-Muhsam (1944) and Hampton (1952), however, have reared pairs of flies with good success.

Materials and Methods

Stock flies were kept in 18" by 18" by 18" cages. The flies fed and oviposited satisfactorily on cellucotton moistened with a mixture of powdered milk and honey. Newly emerged adults were paired and placed in small conical screen cages (figure 3). The cone cage measures 11 cm. in height with a base diameter of 5 cm. and fits into a stender dish of comparable diameter. A cardboard collar is used to prevent intruding flies from ovipositing on the interior of the dish, or the cone may simply be slipped over the dish. Paired and stock flies were fed identically, the former being immobilized daily in a 7° C cold room just long enough to substitute a fresh dish. Knipe and Frings (1953) have pointed out the possibility of a female ovipositing through a screen and dropping her eggs on the substrate which is beyond the reach of her ovipositor. In our experience, even when cone cages were placed in densely populated fly cages, no such adventitious depositions were observed which might be mistakenly attributed to the single female within the cone. Perhaps if the flies in the large cage had not had suitable breeding material, this phenomenon would have been encountered. Egg batches

were found most often tucked away below the surface of the cellucotton in a fairly protected situation. Dishes were examined daily for eggs which were most readily recovered by teasing the cellucotton apart under water. Eggs were then placed on moist paper towelling in a petri dish to hatch.

Cone cages were kept in two types of situations: 1. within the fly cages; 2. within the same room. Throughout these experiments conducted over a period of a year, humidity was uncontrolled and room temperature was maintained near 25° C.

Results

A comparison of the fecundity of pairs of flies kept under the two sets of conditions described above indicates a similar performance for the two groups. Far from demonstrating an inhibiting effect due to isolation, the data show that one of the isolated pairs actually produced almost twice as many eggs as the most prolific control pair. If we omit the data for this unusual pair, the two sets of means show no significant differences, except possibly in the longevity of the male. In the majority of cases, the female outlived the male.

Discussion

There is a striking similarity between these data and those obtained by Hampton (1952). The fact that we used different strains of flies under experimental conditions which must inevitably differ in a number of factors lends support to the results. Feldman-Muhsam (1944), working with the Levant fly, Musca domestica vicina (Macq.), calculated the average daily egg yield per female, disregarding the pre-oviposition period, as 30.6. In this study, it was found to be 37.1, again a fairly close correspondence.

Dunn's data (1923) indicate a rather extreme example of fertility. The selection of large females (about 8.5 mm. in length) would help to account for this phenomenon. Medium sized flies (about 7 mm. in length) usually lay batches containing less than 100 eggs. Though the number of batches produced during their lifetime is comparable to that of large flies, the number of eggs per batch is dependent on the number of ovarioles, which, in turn, may be a function of the individual's size. Under favorable conditions, individuals have fairly consistent maxima, whatever their size. Twenty four hours is approximately the minimum time for the maturation and deposition of a batch of eggs, and the process can be repeated on three consecutive days.

Concerning the relationship of age to egg

production, the data from 11 females have been analysed in the following way. The adult life of the fly, excluding the preoviposition period, has been divided into three equal periods. Since the average longevity was about 25 days, each period would constitute slightly more than 8 days. The number of eggs laid by a fly for each period are tallied and percentages obtained; averages for all the flies for each period are then computed. In this way it can be shown that there is a decline in egg production from 43.4 per cent of the total during the first period, to 34.9 per cent and 21.7 per cent, during the second and final periods, respectively. Roubaud (1922) observed a decrease in egg laying during the second month among populations of flies maintained in large cages. He ascribes this reduction and also the poor fecundity of flies in small cages to a restriction of flight. In the large cages, the increased fraying and tearing of the wings gradually reduces the ability to fly. This hardly suffices as an explanation for our experiments where the cone cages reduce flying to a minimum, without any apparent reduction in fecundity.

Since the visual presence of other flies seems to have no effect on the fecundity of enclosed pairs of flies, it was thought that possibly an olfactory stimulus, originating with the caged flies and

permeating the room, might affect egg laying. Some preliminary experiments were performed in which pairs were kept in rooms where no other flies were present, while controls were kept in the fly room and in the cages themselves. The average preoviposition period for 5 isolated pairs was 5.9 days, compared with 3.7 days for 3 controls, and there was some indication that isolated flies laid fewer eggs, but a quantitative comparison cannot be made at this time.

TABLE VI. COMPARATIVE FECUNDITY OF ISOLATED AND CONTROL FLIES

#	Preoviposition period (days)	Number egg batches	Total eggs	Frequency life/batches	Longevity (days)	
CONTROLS						
1	4.0	4	265	6.0	24	12
2	4.0	9	891	4.1	37	40
3	4.0	-	-	-	-	-
10	3.0	11	772	2.2	24	17
11	2.5	11	821	2.3	25	24
12	2.5	7	772	3.1	22	-
15	4.0	8	837	2.9	23	27
16	4.0	9	768	2.0	18	16
Average	3.5	8.6	732	3.2	24.7	22.7
ISOLATED						
8	4.0	4	459	5.8	23	11
9	2.5	14	1688	2.0	28	24
13	2.5	8	891	3.3	26	22
14	3.0	11	647	2.4	26	20
17	3.0	10	914	2.7	27	12
Average	3.0	9.4	916	3.4	26	17.8

Several studies have been made on the ability of the various stages of the housefly to survive cold, and the subject is well reviewed by West (1952). Wigglesworth (1951) discusses the effect of pre-conditioning on the temperature response of various species of insects. Table 7 summarizes the results of several experiments wherein larvae reared at 21° C, at room temperature (25°C to 28°C), and at 36° C are subsequently exposed to prolonged cooling and then returned to room temperature to complete their development. The eggs used were from pools obtained from many females, and the breeding material was uniformly prepared for both groups. Maggots reared at 21° C lagged about 7 days behind the 36° C maggots in development. When full grown, the maggots were counted, returned to the medium, and placed at 7° C for 6 days. At the end of this time, survivors were counted and returned to room temperature to complete their development. Under normal conditions, 90 to 100 per cent of mature third instars will pupate. Maggots reared at 21° C show no greater ability to survive prolonged cooling than maggots reared at room temperature; but those reared at 36° C have a markedly reduced ability to survive. The physiological basis for this phenomenon of preconditioning remains obscure, but from a behavioral point of view, it may simply be that the conditioned

larvae, having some mobility at the lower temperature, are able to feed and thus remain alive. Eighty four per cent of unconditioned larvae, kept in moist sawdust without food, survived 7° C for 2 days but after 5 days, only 12 per cent were still alive. In nature, the advent of cool weather conditions the cold survival of the larvae and may aid them for a time to avoid freezing temperatures by enabling them to move slowly down into the substrate. Mature maggots uncovered in the fall when the soil temperature was 8° C immediately began burrowing into the soil. Whether this can lead to the survival of maggots through the winter is questionable, but it does emphasize the necessity for considering the factor of preadaptation in laboratory studies on temperature survival.

In line with this idea, it was decided to keep flies outdoors to test their ability to survive low temperatures, under approximately natural conditions. Exposure to low temperatures in laboratory experiments is usually more sudden than it is in nature. A conical cage containing 30 recently emerged flies was placed outdoors on November 9 and maintained through December 15. The flies were fed in the usual manner. During this period there were many days when the minimum temperature was at or below freezing. By December 14, 17 flies were dead. There followed a period of 44 hours during which the average hourly temperature was -5.0° C with a maximum of -1.2° C and a minimum of -8.5° C.

This minimum was recorded for two alternate hours with an intervening temperature of -7.9° C. Seven of 13 flies survived this exposure. The survivors were brought into the laboratory and during the first few hours were poorly coordinated, walked with difficulty and were unable to fly. Two days later, however, oviposition occurred. Another cone cage containing 20 flies, put outdoors on December 6, had only one survivor during the same test period. No significant differences were observed in the cold hardiness of the sexes. On the basis of these data, it is not possible to conclude that the adult is able to survive the winter in this area. More field studies are necessary before any conclusion can be reached.

TABLE VII. SURVIVAL OF VARIOUSLY CONDITIONED LARVAE EXPOSED TO 7°C FOR 6 DAYS

Rearing temp.(C)	Number of survivors	Per cent survivors	Per cent of survivors pupated
36°	12/39	30.7	8.3
36°	9/137	6.5	000.0
36° [6]	20/31	64.5	00.0
25°-28°	299/314	94.9	90.6
21°	19/19	100.0	78.9
21°	271/307	88.3	45.7

[6] Reared at 25° to 28° C and then placed at 36° C for 48 hours before being put in cold room.

Third instar larvae have been found among normal laboratory populations which possessed aberrant mouthparts and darkened cephalic lobes; this latter character readily distinguished them from normal larvae (figures 4,5,and 6). No adults were obtained from the pupae, indicating a possible lethal effect in the pupa stage. The larval mouthparts depart from the normal in that the mouth hooks project straight forward instead of curving ventrad.

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Figure I. pH activity curves for all stages.

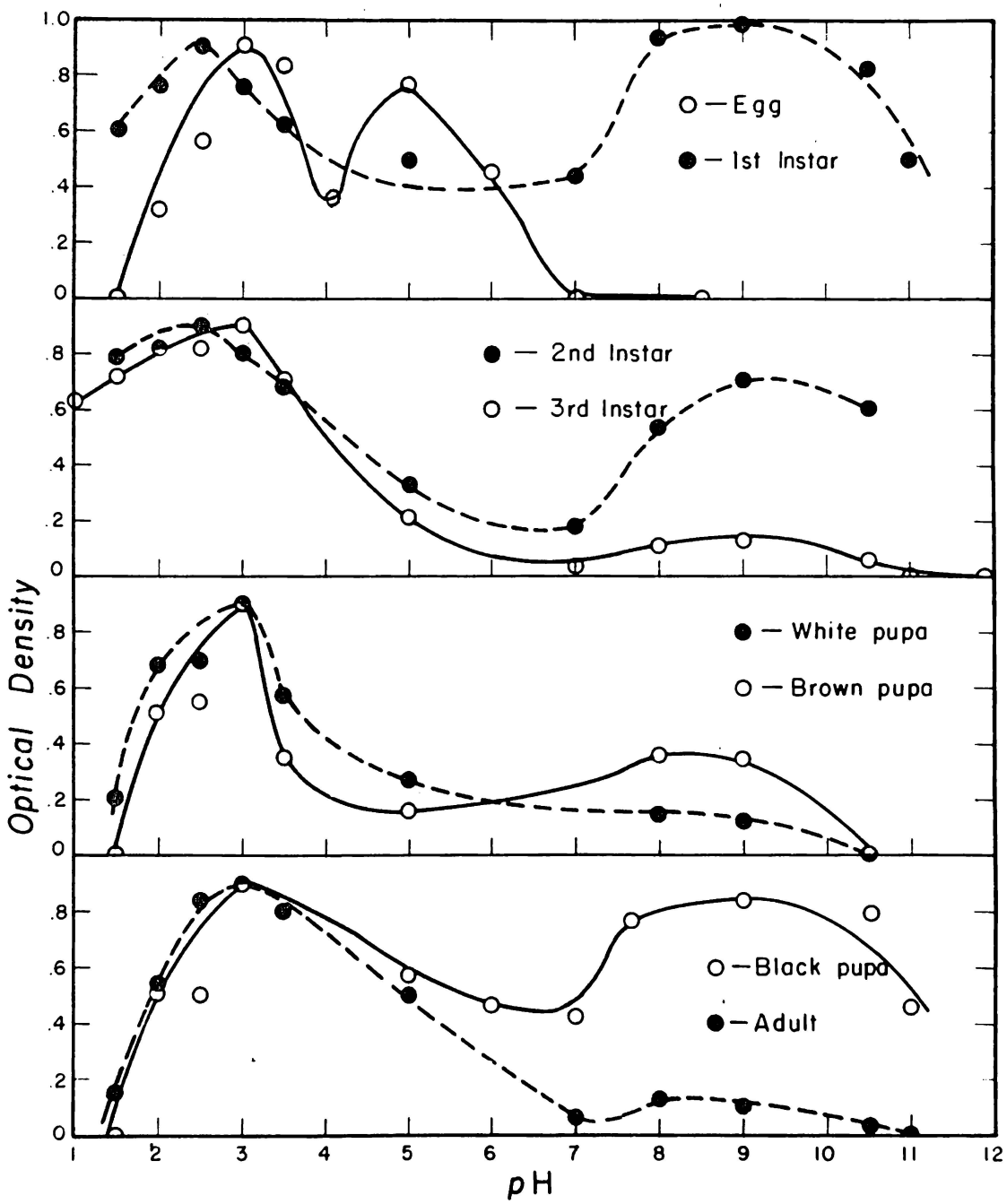
Optical Density
pH
O — Egg
● — 1st Instar
● — 2nd Instar
O — 3rd Instar
● — White pupa
O — Brown pupa
O — Black pupa
● — Adult

Figure 2. Enzyme activity for all stages.

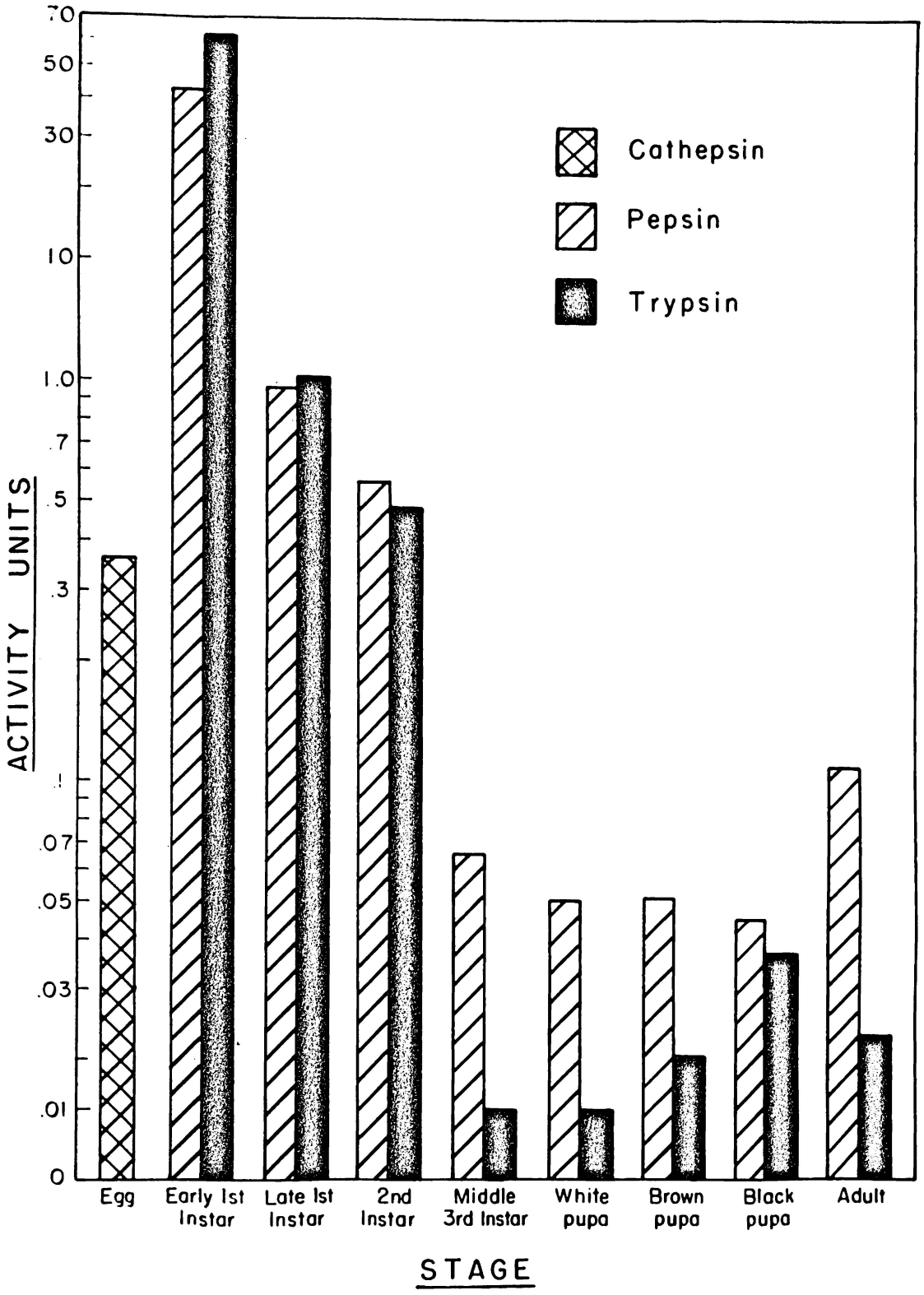
70
50
30
10
1.0
.7
.5
.3
.1
.07
.05
.03
.01
0
ACTIVITY UNITS
Cathepsin
Pepsin
Trypsin
Egg
Early 1st Instar
Late 1st Instar
2nd Instar
Middle 3rd Instar
White pupa
Brown pupa
Black pupa
Adult
STAGE

Figure 3. Conical Cage

Figure 4. Cephalic lobes of normal and aberrant (top) larvae.

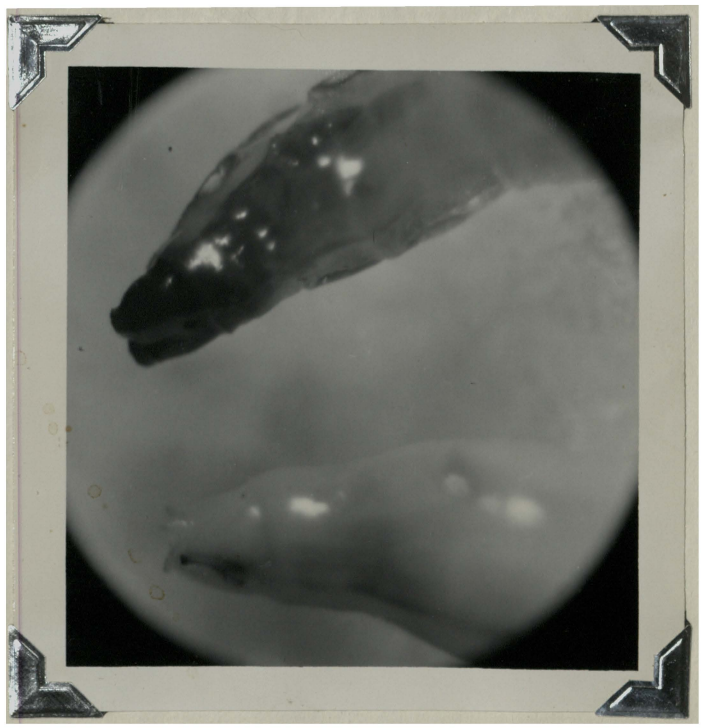

Figure 5. Cephalo-pharyngeal skeleton of aberrant (top) and normal larvae.

Figure 6. Normal (right) and aberrant pupae.