Israel Journal of Entomology Vol VII, 1972

DIET DEPENDENT CYCLING OF PROTEASE ACTIVITY IN THE MIDGUT OF THE FLESHFLY, *SARCOPHAGA BULLATA*[1,2,3]

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ABSTRACT

The time from eclosion to larviposition is 8 to 9 days in *Sarcophaga bullata* reared at 25°C and 60% RH with a 12:12 photoperiod. During this time, females begin feeding on meat at day 3, meat in the midgut reaching a maximum level at day 4 and decreasing until day 6 or 7 when it is no longer visible. Along with feeding, there is an increase in weight on day 4, when, the animals reach a level which remains stable until larviposition begins. Control flies fed on sugar from eclosion to 10 days into adulthood show no increase in weight, nor does larviposit'on take place. Assays of midgut protease made on female sugar fed flies show cyclic activity, beginning on day 3, reaching a maximum and minimum on days 4 and 5 respectively, and starting a second cycle on day 6. Three such cycles may take place within the experimental period of 10 days. Similar assays made on meat fed flies demonstrate a single cycle beginning on day 3 and ending on day 5. No new cycles are instituted for the duration of the experiment. Addition of meat to the diet of sugar fed flies at a time after the peak of a cycle has the effect of terminating cycling. The protease level decreases to a minimal value and remains thus until the experiment ends. These animals do not mature eggs as a consequence of the addition of meat to the diet. The results are discussed in terms of their significance with respect to control of natural feeding behavior of *Sarcophaga* species.

1. The above named students and faculty of the Department of Entomology are pleased to be given the opportunity to contribute this article in honor of Professor G.S. Fraenkel's 70th birthday. His pioneering research in the field of insect nutrition has left us a legacy of important and exciting questions for which we shall long be in his debt.
2. Please address reprint requests to S. Friedman.
3. This work was partially supported by NIH grant AI-06345 to S. Friedman.

INTRODUCTION

An elucidation of the mechanisms that regulate the synthesis and release of insect midgut proteases has been the concern of a number of investigators for the past ten years. At present, direct proof of the existence of any particular control scheme remains elusive, but enough indirect evidence is available to suggest that there is probably no single mechanism operating in all insects.

Among the earlier claims of factors affecting protease activity is that of Dadd (1961) who demonstrated that activity in the midgut of *Tenebrio molitor* adults was responsive to a humoral factor originating in the head. This was extended by Thomsen and Møller (1959, 1963) who reported that surgical removal of the medial neurosecretory cells from the brain of *Calliphora erythrocephala* results in a depression of proteolytic activity in the midgut of these flies. The possibility of neurosecretory control has also been recently examined by Dogra and Gillott in *Melanoplus sanguinipes* (1971). Using autoradiographic methods they have observed that when previously starved grasshoppers are fed, neurosecretory material moves rapidly from the neurosecretory cells along the nervi corporis cardiaca internus to the corpora cardiaca. Further studies have correlated this neurosecretory movement with a decrease in blood protein level and an increase in protease activity during the first day of feeding.

Langley (1966, 1967) has extended the neuroendocrine hypothesis, stating that in the tsetse fly *(Glossina morsitans)*, stretch receptors along the crop wall are stimulated by the ingestion of a volume of food, and act in turn to initiate the release of a hormone from the neurosecretory cells. This hormone, according to Langley, acts upon the midgut cells, causing the synthesis of an inactive form of the proteolytic enzyme which is later activated in the lumen of the gut by some specific factor present in the food. The bulk of Langley's proposal is supported by Yang and Davies (1968) who found that trypsin activity in the midgut of three species of black flies was dependent upon the amount of a blood-sucrose mixture that was ingested.

In contrast to neuroendocrine involvement, a "secretagogue" hypothesis has been proposed for the cockroach, *Leucophaea maderae* (Engelmann, 1969) and the fleshfly, *Sarcophaga bullata* (Engelmann and Wilkens, 1969). The basic premise of Engelmann's hypothesis is that the presence of proteinaceous food in the midgut specifically elicits the synthesis and secretion of proteases. The level of protease activity is supposed by Engelmann to be dependent upon the nature of the meal.

The present study was begun as an attempt to evaluate the effect of diet on protease production in *Sarcophaga bullata*. The paper by Engelmann and Wilkens (1969) appeared while this work was in its early stages, but our approach seemed sufficiently different to warrant continued investigation. The results presented herewith bear out the correctness of that decision.

METHODS AND MATERIALS

Experimental Conditions

Adult female *Sarcophaga bullata* were used for all experiments, each sample population consisting only of those flies emerging from puparia within a 24 hour period. These flies were kept in cages measuring 2' x 2' x 3' and held at 25°C and 60% relative humidity. The photoperiod was maintained on a 12:12 light-dark cycle.

Fresh food and water were provided daily for all cages. Food for the meat-fed or meat-sugar fed flies consisted of pork liver homogenized to a paste either in distilled H_2O (feeding choice experiments), or in sucrose (5% w/v) in distilled H_2O (other experiments as noted). The diet of sugar-fed flies consisted solely of five percent sucrose in distilled H_2O. Dead flies were removed from each of the cages twice daily, and the floors and walls of the cages containing the sugar-fed flies were cleaned with a wet sponge each day. These measures were carried out to prevent the sugar-fed flies from obtaining protein either through cannibalism or fecal feeding.

Dissections

Prior to dissection, flies were removed from the cages and immobilized by chilling on ice. Each fly was weighed to the nearest tenth of a milligram on a Roller-Smith torsion balance and the midguts were dissected out in an isotonic saline solution and cleaned of adhering malpighian tubules. An estimate of the meat content of the gut was then made, always by the same observer, using as a measure the relative distension of the midgut with the dark colored mass. After evaluating the gut contents, the midguts were immediately used for the enzyme assays.

Enzyme Assays

Midguts were removed from female *S. bullata* adults fed on sugar or meat-sugar for protease activity determinations at the same time each day. The guts of animals fed on meat-sugar were rinsed to remove the meat prior to homogenization. Whole midguts were placed into a glass tissue grinder and homogenized in 1.0 ml of phosphate buffered saline, pH 7.0 (Miller, 1968). The assays for protease activity were performed using Hide Powder Azure, B grade (Calbiochem) as the substrate. This substrate, an insoluble protein-dye complex, has been reported by Rinderknecht, *et al.* (1968) to be an extremely sensitive means of determining proteolytic activity. Each assay was run in a 10 ml erlenmeyer flask, the mixture consisting of 0.1 ml of the homogenate, 0.9 ml of buffered saline and 20 mg of substrate. The substances were gently agitated in a water bath at a temperature of 37°C for 15 minutes and the reaction stopped by placing the vessel into an ice bath. (Preliminary experiments had shown that chilling in an ice bath sufficiently stopped any further hydrolysis of the substrate.) The reaction mixture was then centrifuged at 10,000 x g for five minutes in a refrigerated centrifuge at 1°C and the supernatant liquid decanted into a cuvette for colorimetric assay in a Zeiss PMQ-II spectrophotometer set at 595 nm. Blanks were treated in the same way except that the homogenate was not added to the reaction mixture. Protease activity is expressed in terms of optical density (O.D.) units/0.1 ml of gut homogenate.

RESULTS

In order to assess the normal feeding activity of flies, newly emerged female *Sarcophaga bullata* were placed in cages containing meat and sugar in separate dishes. The results, based on the observed presence of meat in the midgut, showed that feeding on meat did not begin until the third day after emergence. The quantity of meat in the midgut reached a maximum on day four and then decreased until day six or seven when no meat could be seen. The gut then remained free of meat for the duration of the ten day experiment.

An attempt to induce early feeding on meat by mixing the meat in a sucrose solution was successful, in that the midguts of all flies thus treated were distended with meat within 24 hours after emergence and remained in this condition for four days. The degree of distension was nearly identical for each of the 10 replicates examined every day. By day six, the quantity of meat in those animals whose food source was meat-sugar had decreased to a point where only a small amount was present, but from days seven to ten there was a steady increase until full distension again occurred.

A comparison between the changes in body weight of meat-sugar fed flies and sugar-fed flies is shown in Fig. 1. Sugar fed flies maintained a relatively constant weight for the ten days of the experiment. Meat-sugar fed flies remained at weights approximately equal to the sugar-fed flies for the first three days after eclosion. Their weight increased on day 4 by 15-20 mg, remained at this level for four days, and decreased on days 8 and 9 to the sugar fed level. The presence of larvae on the meat was noted beginning on the eighth day. A measure of the number of larvae per female and the weight of a single larva revealed that each female averaged 56 larvae weighing approximately 0.27 mg apiece. Therefore, the average weight of a complement of larvae was approximately 15 mg which is almost exactly the mean weight loss between days seven and eight. Other experiments conducted in the same way gave similar results, the increase in weight of the meat-sugar fed flies occurring between days 3 and 4. Determinations of protein in washed midgut homogenates of meat-sugar and sugar fed flies show an interesting relationship to the above experiments. As seen in Fig. 2, values in meat-sugar fed animals reach a maximum at four days after eclosion, declining on day 5 and remaining low thereafter.

The assays for midgut protease activity were designed to determine what effect the presence of meat in the midgut had on the activity of these enzymes. Female flies were tested at 24 hour intervals beginning on the first day after emergence. Ten flies on sugar and on meat-sugar were used every day for the assays and the results, shown in Fig. 3, are expressed as a mean for each group.

Those flies maintained on a diet regimen consisting solely of five percent sucrose demonstrated two complete cycles of protease activity, indicating that the enzymes are present even in the absence of a protein source. The actual number of times that the proteases cycled during the ten days of the experiment was found to vary between two and three depending on whether the first cycle peak was on day two or day three. This is seen in Fig. 4. Meat-sugar fed flies also showed the presence of proteolytic enzymes; however, only one complete cycle of activity was observed. This experiment was repeated three times under similar environmental conditions with the same results. The protease activity in flies maintained in the absence of a protein source

continuously cycled, whereas those flies that were allowed to feed on meat exhibited only one cycle within the ten days of observation. T-test comparisons and T' approximations (Sokal and Rohlf, 1969) indicated that the peaks and valleys of each cycle were significantly different below the 5% level and that the curve derived from the results of the sugar fed flies was different from that obtained from meat-sugar fed flies.

The questions occasioned by the above results were two: If meat-sugar was fed to flies previously fed on sugar would it stimulate or depress protease activity?, and, Would flies previously fed on meat-sugar show a recycling of proteases if meat was eliminated from their diet? The results of such a diet substitution experiment are shown in Fig. 5. When flies were fed on sugar to the end of a cycle and then changed to meat-sugar the result was rapid decline in protease activity to a low level. Those flies that were fed on meat-sugar for six days and then changed to sugar did not initiate a second protease cycle. The dissections also revealed that those flies that were fed meat-sugar until day six produced normal larvae, whereas those flies that were fed on meat-sugar after day six showed no change in the development of their ovaries through day 10 as measured by the average lengths of individual eggs. Sugar-fed flies behaved as did the latter group, i.e., they produced eggs that did not mature during the ten days of the experiment. The average length of the non-maturing eggs did not exceed 0.20 mm, whereas the average length of the mature eggs in the continuously meat-sugar fed flies was 1.8 mm.

DISCUSSION

The results obtained in the feeding choice experiments demonstrate that *Sarcophaga* females begin to feed on carbohydrate relatively soon after emergence. However, it is not until a number of days have passed that receptivity (sensitivity?) to protein begins to manifest itself. An examination of the gut at various times after eclosion is of some interest, because using color and turgidity as an indicator of the presence of meat, we found that feeding on meat is cyclic, a visible quantity being present only from days 3–5. Adding this information to that obtained from the analyses of protease activity, protein, and fresh weight leads us to conclude that there is an extremely tight coupling between the appearance of preference for (sensitivity to?) protein, and the appearance of protease. It seems that all of the activity with respect to uptake and incorporation of amino acids into a form which will be used for oocyte and larval production takes place between days 3 and 5. The average fresh weight of the females increases by more than 15 mg within a single day and remains at this level until larviposition takes place; the protease activity and protein content of the gut are back down to a low level within a day; and, as stated before, the meat is completely gone from the midgut within two days.

A second conclusion, also noted by Wilkens (1968), is that feeding as a whole is cyclic in meat-fed females. When flies were forced to ingest meat in order to obtain sugar, there was a marked drop in the amount of meat in the gut on days 5–7, indicating a decreased intake of sugar as well as no intake of meat during this time.

Protease analyses show that cycles of proteolytic activity apparently occur independent of dietary protein in the guts of female *Sarcophaga* adults. Meat seems to exert a measure of control over cycling, being inhibitory in nature, but an examination

of the data argues for a limited capability. The results expressed in Fig. 5 show that putting meat into the gut at the end of a protease cycle prevents new cycles from appearing and leads to no ovarian development. However, the presence of meat at the beginning of a cycle, as may be seen in experiments in which it is "force-fed" from day 1, does not prevent the first cycle from taking place, and a recent incompletely analyzed experiment in which meat was given to sugar-fed animals just prior to the beginning of the second cycle shows a similar lack of inhibition. Indications are that depending upon the temporal state of the program controlling the cyclic behavior, the meat will or will not inhibit the following cycle.

If the above experiments are analyzed in terms of the animal in nature, it is obviously undesirable that meat should get into the gut at the end of a protease cycle in which the meat "hunger" has not been assuaged, because new cycles would then not appear. But, if the assumption is made that sensitivity to protein and protease production are cyclic and tightly coupled, it is equally obvious that this would never normally occur, since the sensitivity to protein at the end of a cycle would be so low that there would be no feeding on proteinaceous material, and the animal could not be induced to feed on protein until such a time that protease synthesis was already in a stage in which the next cycle could not be inhibited.

However, the fact that meat, experimentally put into the gut at a certain time, can inhibit further protease cycling without being utilized for egg production, permits the conclusion that ovarian activity need have no input into the regulation of cycling.

Evidence has been presented by Thomsen and Møller (1963) that cycles of enzyme activity also occur in the midgut of *Calliphora erythrocephala*. In *Calliphora*, both sugar-fed and meat-fed females reach a maximum level of protease activity on the third day after emergence. Unfortunately, their observations only extended for five days and no mention was made of any recurrent cycling.

A cycle of protease activity has been recently cited by Persaud and Davey (1971) to occur in *Rhodnius prolixus* beginning within 24 hours after the initial blood meal and reaching a peak of activity on the fourth day. They could find no evidence for an endocrine involvement nor could they demonstrate any connection between the protease activity cycle and egg production. This has prompted them to conclude that the secretagogue hypothesis of Engelmann best fits with their observations.

The problem of the synthesis of digestive enzymes in *Sarcophaga bullata* has been studied by Engelmann and Wilkens (1969), and they have reported that the level of protease activity in the midgut is directly proportional to the amount of meat ingested by the female fly, sucrose being completely ineffective in stimulating enzyme activity. It is interesting in this regard to note that their experiments were performed on flies which were four days old and had been starved for two days. Whether this treatment is enough to upset the cyclic activity we have shown can only be determined by further investigation. Certainly our results with the sugar fed flies unequivocally demonstrate that cycles of proteolytic enzyme activity occur independently of any "secretagogue".

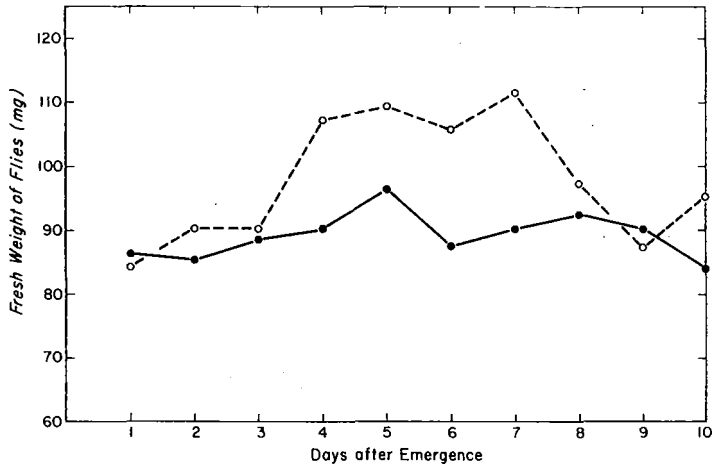

Fig.1. Influence on diet on fresh weight of adult female *S. bullata.* Each point is an average of 5 individual flies weighed as designated in the Materials and Methods section. (● - - ● sugar fed flies; o - - o meat-sugar fed flies.)

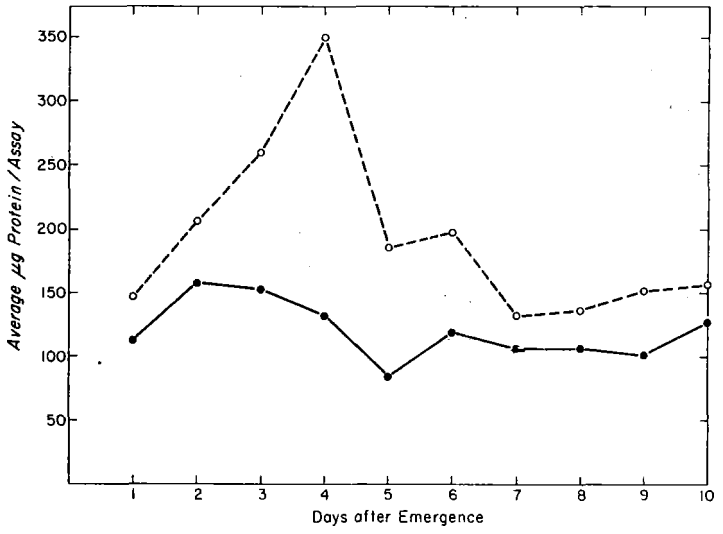

Fig. 2. Influence of diet upon protein level of adult female *S. bullata* midgut. Midguts washed with saline and homogenized as described in Materials and Methods section. Protein assay according to Lowry, et al. (1951). (● - - ● sugar fed flies; o - - o meat-sugar fed flies.)

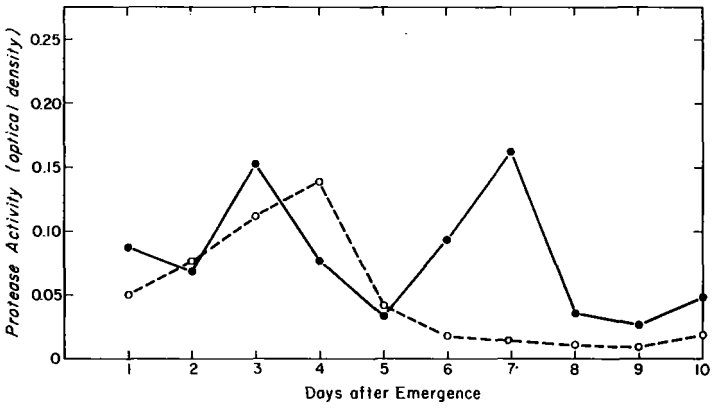

Fig. 3 Influence of diet on adult female *S. bullata* midgut protease levels. Each point is an average of 10 individual flies assayed as designated in the Materials and Methods section. See text for summary of statistical treatment. (● - - ● sugar fed flies; o - - o meat-sugar fed flies.)

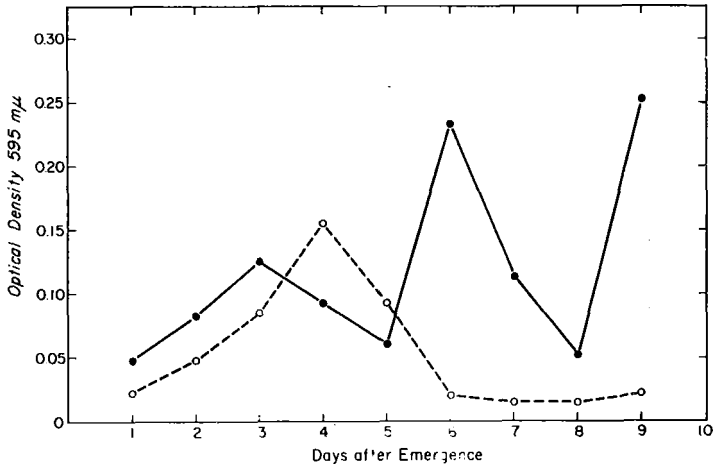

Fig. 4 Influence of diet on adult female *S. bullata* midgut protease levels. (Experiment carried on for nine days. See Fig. 3 for details.)

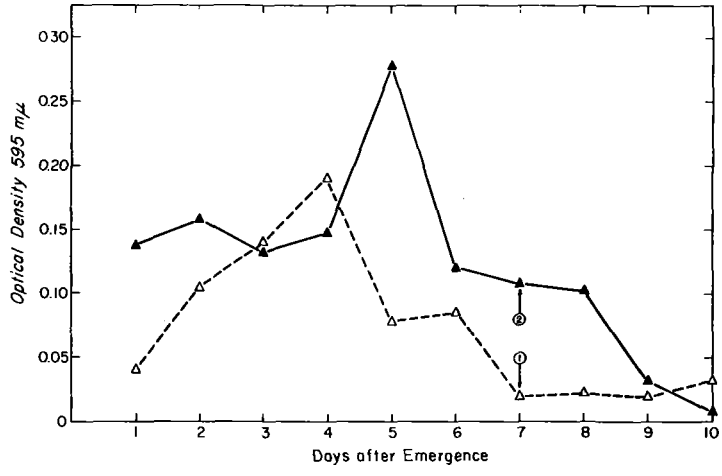

Fig. 5 Effect of diet substitution on midgut protease levels of adult female *S. bullata.* See Figure 3 for details. (△ - - △ meat-sugar fed changed at ① to sugar-fed; ▲- - ▲ sugar fed changed at ② to meat-sugar fed.)

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