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Insectivory and Social Digestion in *Drosophila*

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It has long been believed that Drosophila *larvae feed almost entirely by ingesting yeast and possibly other microorganisms that are associated with fermenting fruits or other vegetable matter. However, we have discovered that the larvae of a number of* Drosophila *species can consume such diverse substrates as insect tissues, including the exoskeleton. Experiments reported here, which include raising sterile dechorionated eggs to adulthood on adult carcasses under axenic conditions, show that larvae can consume complex chitinous substrates directly without the assistance of microorganisms. We show that* Drosophila *larvae are able externally to digest amylose, cellulose, and chitin, without coming into physical contact with them. We conclude that not only do* Drosophila *larvae produce enzymes enabling them to digest a wide variety of substrates, but also these enzymes are egested onto the substrates so that at least some digestion, especially of large polymers, takes place externally. Finally, we suggest that the phenomenon of external digestion explains both the previously unexplained massiveness of* Drosophila *salivary glands and their chromosomes and the tendency of larvae to cluster, which may also be true of other dipterans.*

KEY WORDS: cellulase; chitinase; *Drosophila hydei;* external digestion; social digestion.

INTRODUCTION

The experiments reported here were prompted by an earlier observation (Rypstra and Gregg, 1986) that *Drosophila hydei* larvae could be reared to adulthood more quickly on insect carcasses than on laboratory culture media and that larvae so reared developed into full-sized normal adults. In that study

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900 mg of cicada carcass supported the development of 90 mg of *D. hydei*. This argued for at least some direct consumption of insect tissues by the larvae, since by standard assumptions about conversion efficiencies there was not room for another complete trophic level, e.g., microorganisms, between the carcass and the adult *Drosophila*. In this same study the possibility of external digestion was raised by the observation that more delicate substrates, such as the carcasses of *hydei* adults, became thoroughly moistened by larval activity and appeared to dissolve. If our conclusion about larvae producing enzymes for the direct consumption of carcasses was correct, external digestion seemed likely. However, the common view is that both the consumption of insect tissues, especially chitin, and the moistening and dissolution of carcasses, although surprising, must be due to microbial activity. Standard references to digestive enzymes (Vonk and Western, 1984) and to insect digestion and nutrition (Wigglesworth, 1972) cite a few cases of insects producing chitinases and/or cellulases and a few instances of external digestion, but not together, and especially not in *Drosophila*. On the other hand, studies of *Drosophila* nutrition have concentrated almost exclusively on yeast and other microorganisms (Begon, 1982). Although chitinase has been shown to play a role in molting in *Drosophila* (Winicur and Mitchell, 1974), it has not, to our knowledge, been invoked as a digestive enzyme or as being involved in external digestion. The standard *Drosophila* reference (Ashburner *et al.*, 1976, *et seq.*) does not mention either of these possibilities, nor do two monographs specifically dealing with unusual feeding habits and breeding sites in *Drosophila* (Ashburner, 1981; Lachaise and Tsacas, 1985). Studies on the midgut enzymes of caddisflies (trichoptera) (Martin *et al.*, 1981), and blackflies (diptera) (Martin *et al.*, 1985) did not disclose the presence of either chitinase or cellulase activity. Cooperative external digestion has been suggested for social spiders (Krafft, 1971) but has not been definitively demonstrated.

In addition to the observation that *hydei* larvae appear to be moistening and dissolving carcasses, other observations supporting the hypothesis that *Drosophila* larvae engage in external digestion are as follows.

(1) The salivary glands, which are connected to the pharynx immediately adjacent to the mouth by a common duct, are many times larger than the combined size of the pharynx, esophagus, stomach, and gastric cecum which, under standard assumptions, they ostensibly would serve.

Of course, for the large size of the salivary glands to be an argument for external digestion, one must assume that they produce digestive enzymes, an assumption not agreed to by everyone. *Drosophila* salivary glands have been extensively studied and reviewed (Ashburner and Berendes, 1978), as have the puffing patterns of their giant chromosomes (Berendes and Ashburner, 1978). Yet the only product of the salivary glands so far identified and studied has been the glue used in cementing the puparium to the substrate. It has been

argued (Ashburner and Berendes 1978; Ashburner, personal communication) that the salivary glands do not, in fact, produce digestive enzymes.

(2) On laboratory media larvae cluster together in masses in both uncrowded and overcrowded cultures. This clustering, which also occurs among the larvae of flesh flies, screw worm flies, and undoubtedly other dipterans, appears to be a normal behavior and one that is consistent with the hypothesis that it evolved to increase the efficiency of external digestion.

(3) A few thousand washed larvae from a healthy culture, when put into a medium bottle covered with a 0.25-in.-thick blanket of fungi, can completely destroy the fungi within a matter of hours (overnight). *Drosophila* larvae do not have chewing mouth parts and there does not seem to be any way that they could be individually devouring such a large volume of fungi in such a short time.

(4) Second- or early third-instar *D. hydei* larvae, when provided with pupae of their own species as a sole food source, moisten the opercular area of the puparium, force the operculum open, and consume the puparium so completely and cleanly that it does not seem possible that they could have accomplished it just by rasping with their mouth hooks.

(5) When one has several larvae in a watch glass to dissect out salivary glands and one of them has been ripped apart, the others quickly converge on the remains, mouth parts working furiously. Even though they do not ingest particulate debris, this apparent feeding frenzy strongly suggests that the consumption of predigested substrates is typical and that liquefied insect tissues and hemolymph are delicacies. The latter two observations, 4 and 5, also reinforce the idea that the consumption of insect tissues by *Drosophila* larvae is not unusual.

In this report we offer direct evidence that *Drosophila* larvae can digest amylose, cellulose, and chitin without coming into direct contact with these substrates, and without the aid of microorganisms. The choice of substrates is explained below.

MATERIALS AND METHODS

Substrate Sandwiches

The hypotheses of external digestion and the presence of specific enzymatic capabilities were tested simultaneously. To do this the various substrates to be tested were sandwiched between two 47-mm filter disks of paper, glass, or nylon, in the top of an inverted 35 × 10-mm Falcon tissue culture dish. Substrate suspension was pipetted onto the bottom filter and the top filter placed over it. Approximately 700–900 washed larvae, along with any sustaining additives such as autoclaved yeast or bovine serum albumin, were then

placed on the top filter. The larvae were obtained from cultures in which most of the individuals were in the late second and early third instar. They were collected by washing the entire contents of a half-pint culture bottle through a seive and transferring the larvae which were retained in the sieve to a 4000-ml beaker, where they were swirled in 4000 ml of tap water and allowed to settle to the bottom. The water was then carefully decanted. This was done three times. The larvae were then recollected in the sieve and transferred to the filters with a spatula. The bottom of the Falcon dish was then fitted snugly inside the top, squeezing the edges of the filter disks together so that larvae could neither get between the filters nor escape. Thus, the bottom filters contained the substrates, which were protected from direct contact with the larvae by the top filter (for filter thicknesses and pore sizes see below). A small hole in the Falcon dish allowed for gas exchange and the addition of water, as needed, to keep the dishes moist. Although these conditions, including washing, must have been harsh, there was very little mortality. After 1, 2, or 3 days the bottom filters were monitored for the disappearance of substrate or the appearance of product. To ameliorate the harsh conditions autoclaved yeast was added to the top filter as a food source in the earlier experiments. This did not pose a problem in the disappearance experiments, but it did pose a problem with those in which the appearance of product was measured because larvae were able to produce, or otherwise free, reducing sugars from the yeast. Those experiments were done first with no supplement and then with a bovine serum albumin supplement which does not interfere with the assay.

Filters

At first Whatman No. 1 filter paper disks were used, but when we began to suspect the larvae of digesting the cellulose, we switched to glass and nylon. Paper filters did not confound the experiments in which disappearance of product was monitored, but it did in those monitored for appearance of product. Filter specifications were as follows: paper—Whatman No. 1, 11-μm pore size, 160-μm thickness; glass—Micron Separations Inc., 1-μm pore size, 280-μm thickness; and nylon—Micron Separations, Inc., 10-μm pore size, 75-μm thickness. The paper and glass filters were much more absorptive than the nylon filters.

Substrates

The substrates tested were amylose (electrophoresis-grade potato starch, Sigma Co.), clam shell chitin (Sigma Co.), and subsequently, cellulose (microcrystalline cellulose, Polysciences, Inc.), to test for amylase, chitinase, and cellulase activities, respectively. We tested for amylase activity because

amylase is such a common digestive enzyme and it is easily detected by standard assays. We tested for chitinase activity for reasons discussed above and for cellulase activity because we came to suspect its presence because of the ease with which larvae destroyed paper filters in the early stages of these experiments.

Monitoring the Effects of Larvae on Substrates

The presence, and thus the disappearance, of all three substrates could be monitored by inspection after adding a few drops of Lugol's solution (1% iodine in water) to the bottom filter. Amylose turns blue and can be observed with the unaided eye. Chitin stains yellowish-brown and was observed under a dissecting microscope at 60× magnification to distinguish between chitin particles and background yellowing of the filters. Microcrystalline cellulose, when simultaneously treated with Lugol's solution and 65% sulfuric acid, produces tiny black particles which also were observed at 60× magnification. The amount of substrate on the bottom filter of control and experimental plates was visually estimated, and the amounts designated with a series of +'s. Thus (++++) was equal to the control value, (+++) had less, and so on. A (−) indicated that no substrate was detectable.

In addition to testing qualitatively for the disappearance of substrate, we tested quantitatively for the appearance of product. The breakdown products of all three substrates are reducing sugars, ultimately glucose from amylose and cellulose and *N*-acetyl glucosamine from chitin, but intermediate products can be reducing as well. These reducing sugars can be quantified spectrophotometrically by reacting them with dinitrosalicylic acid (DNS) (Bernfeld, 1955). This was done by putting the bottom filters into 2 ml of acetate buffer (*p*H 4.8) in a 15-ml conical centrifuge tube, adding 2 ml of DNS solution, and boiling for 10 min. This mixture was centrifuged at 1000 rpm for 10 min to clear it of debris. Two milliliters of supernate was further microfuged for 6 min at 9000 rpm in an Eppendorf 5415 microfuge. This solution was read in a Varian DMS 100S dual-beam spectrophotometer at 540 nm against an appropriate blank. Absorbances were converted to maltose equivalents, in grams per filter, by constructing a standard curve with maltose. Differences in mean absorbances were compared using a Mann–Whitney *U* test.

Controls

Controls in the disappearance-of-substrate experiments consisted of substrate sandwiches to which microorganisms isolated from *Drosophila* cultures were added in the absence of larvae. This was to demonstrate that microorganisms

inadvertantly introduced into the experimental dishes along with the larvae were not responsible for the disappearance of substrate.

Controls for the appearance-of-product experiments also included substrate sandwiches to which microorganisms had been added in the absence of larvae to assess the contribution of microorganisms to the appearance of product. A second control consisted of adding larvae to sandwiches in the absence of substrate. This controlled for the possibility that something about the larvae themselves, their excretions, or their interaction with contaminating yeast or other microorganisms might be responsible for the presence of reducing sugars.

Raising Larvae Axenically

Finally, we sterilized and dechorionated *D. hydei* eggs in a 10% Clorox solution for 8 min. These were then rinsed in sterile water and placed on autoclaved adult carcasses in a sterile Falcon dish, maintaining sterile conditions. These cultures required watering with sterile water every 4–6 days. At each watering and at the end of the experiment a few carcasses were plated out on bacterial tryptic soy agar plates to check for bacterial contamination.

Other Species

Several other species were tested for the willingness of females to lay eggs on adult carcasses and for the ability of larvae to develop normally on this food source. The carcasses were autoclaved but no further attempt was made to keep the cultures axenic.

RESULTS

The results of the disappearance-of-substrate experiments are shown in Table I. There was some overlap between the experimental groups and the control groups in that a few controls had reduced amounts of substrate and a few experimental dishes showed no reduction in the quantity of substrate remaining. The controls with reduced amounts of substrate were most likely due to human error resulting from the fact that the substrates settle out of suspension fairly quickly. The variability in the experimental groups was most likely due to variability in larval behavior. Nonetheless, of 110 controls, only 6 had noticeably less than the standard amount of substrate, whereas in sharp contrast, among the experimentals 117 of 126 had decidedly less substrate, or none at all, remaining on the bottom filter. Moreover, Table I shows, there is a striking difference between the experimental and the control dishes in each category.

Table I. Disappearance of Substrate[a]

Sandwich type	Substrate					
	Amylose			Cellulose	Chitin	
	Paper	Glass	Nylon	Glass	Glass	Nylon
Control 1 (yeast only)	3 ++++	14 ++++	5 ++++ 1 ++	38 ++++ 1 ++	2 ++++ 1 ++	27 ++++ 3 ++
Control 2 (yeast, and microorganisms in excess)	3 ++++	6 ++++			6 ++++	
Experimental 1 (larvae and yeast added)	2 ++++ 2 ++ 3 −	3 ++++ 24 ++ 15 −	15 ++	2 ++++ 11 ++ 3 −	10 ++ 1 −	2 ++++ 22 ++ 11 −

[a]The substrates shown were sandwiched between the filters shown, as described in the text. Sandwich types differ in terms of what was added to the top filter. Substrate suspension was pipetted onto the bottom filter as follows: 0.2 ml of 1.0% (w/v) amylose (potato starch), 0.2 ml of 0.5% (w/v) microcrystalline cellulose, and 0.2 ml of 0.5% (w/v) clam shell chitin. Amounts of substrate remaining were visually estimated after staining with Lugol's solution (see text). (++++) The amount of substrate normally found in the control; (++) a distinctly smaller amount than found in normal controls; (−) no detectable substrate.

The results of the appearance-of-product experiments are shown in Table II. We were unable to get conclusive results with cellulose because the substrate itself reacted too strongly with DNS, so only the chitin and amylose results are shown. Prior to this set of experiments we had abandoned yeast as a sustaining supplement because we belatedly discovered that larvae were releasing reducing sugars from yeast, an observation which is in itself indicative of external digestion since neither yeast alone nor larval feces react strongly with DNS (data not shown). In the course of the chitin experiments shown here we switched from no sustaining supplement to bovine serum albumin (BSA) as a sustaining supplement which, along with any of its breakdown products, is completely inert with respect to DNS. Thus there are two experimental chitin groups (columns 5 and 6), one with and one without BSA added to the top filter. The differences between the experimental groups and the controls were significant in every case. Again, we conclude that the higher concentrations of reducing sugars in experimental dishes are due to the egestion of digestive enzymes by larvae.

Table II. Appearance of Product[a]

	(1) Larvae with BSA but no substrate ($n = 58$)	(2) Amylose with BSA and microorg. but no larvae ($n = 14$)	(3) Amylose with BSA and larvae ($n = 23$)	(4) Chitin with BSA and microorg. but no larvae ($n = 8$)	(5) Chitin with larvae but no BSA ($n = 19$)	(6) Chitin with larvae and BSA ($n = 16$)
Absorbance	0.129 ± 0.096	0.204 ± 0.081	0.328 ± 0.154	0.151 ± 0.092	0.271 ± 0.144	0.507 ± 0.17
Maltose equivalents in grams per filter*	0.33×10^{-4} $\pm$ 0.76×10^{-4}	0.91×10^{-4} $\pm$ 0.64×10^{-4}	1.9×10^{-4} $\pm$ 1.2×10^{-4}	0.49×10^{-4} $\pm$ 0.73×10^{-4}	1.5×10^{-4} $\pm$ 1.1×10^{-4}	3.3×10^{-4} $\pm$ 1.4×10^{-4}

[a]In all instances the bottom filter was a more absorbant glass filter, and the top filter a less absorbant nylon one (for filter specifications and experimental details see text). In column 1 no substrate was added to the bottom filter, whereas in columns 2 and 3, 0.2 ml of 1% amylose substrate was pipetted onto the bottom filter, and in columns 4, 5, and 6, 0.2 ml of a 0.5% chitin solution was added. Larvae, BSA (bovine serum albumin), and microorganisms were added to the top filters as noted in the column headings. Absorbances are in maltose equivalence per filter. Columns 1 and 2 served as controls for column 3, and columns 1 and 4 served as controls for columns 5 and 6. The experiments in column 5 lacked BSA because we had not yet adopted it as a nutritional supplement to ameliorate the stress of the experimental environment. Numbers given are means and standard deviations.

*Mann–Whitney U test comparisons on grams per filter: Column 1 vs 3, $P < 0.0002$; Column 2 vs 3, $P < 0.0039$; Column 1 vs 5, $P < 0.0002$; Column 4 vs 5, $P < 0.0129$; Column 1 vs 6, $P < 0.0002$; Column 4 vs 6, $P < 0.0002$.

The results of the experiments in which sterility was successfully maintained were variable. In some sterile cultures adults successfully emerged. However, the eclosion rate was approximately 10% and the length of time to eclosion was about 26 days, 10 days longer than if microorganisms are present. In other cases most of the larvae developed to the late third-instar stage but failed to pupate. Obviously microorganisms must play an important role in normal larval development on carcasses and, undoubtedly, on other substrates as well. But just as obviously the larvae themselves produced enzymes that allowed them to live and grow on these chitinous carcasses.

In the experiments with other species it was found that females of *D. melanogaster, D. robusta, D. immigrans, D. putrida, D. tripunctata,* and *D. neohydei* will also lay eggs on adult carcasses and that these eggs develop normally into adults. Of the species tested, only *D. naragannsett* failed to utilize adult carcasses.

DISCUSSION

Since substrate does not disappear spontaneously in any of the experiments (control 1, Table I) or under the influence of microorganisms in the absence of larvae (control 2, Table I), we conclude that its degradation and disappearance in the experimental groups are the result of larval activity, i.e., the egestion of enzymes. The same conclusion is reached in the appearance-of-product experiments (Table II). Larvae supplied with substrate produce significantly more product than larvae without substrate and significantly more product than microorganisms alone. Adding *p*H paper to the top filter indicates a *p*H of 8–8.5, ruling out a generalized acid hydrolysis of the substrates. These results complement the experiments in which larvae were successfully grown on adult carcasses under axenic conditions.

These results taken together provide very strong evidence for the external digestion of amylose, cellulose, and chitin by *Drosophila* larvae. Although we have not directly proven that these enzymes are produced by the salivary glands, their exceptionally large size virtually forces one to this conclusion, despite arguments to the contrary (Ashburner and Berendes, 1978). Studies of specific *Drosophila* enzymes usually have not considered where they are produced (see O'Brian and MacIntyre, 1978). Thus, we contend that one mystery of the giant salivary glands is solved. They need to be large because at least some of the digestive enzymes they produce are required for external digestion and would therefore need to be produced in larger quantities than digestive tract enzymes to counteract the effects of dispersion in the external environment. This conclusion, coupled with the fact that *Drosophila* larvae normally and willfully associate in clusters, strongly suggests that the clustering behavior has evolved to facilitate external digestion. We suggest the term

"social digestion" to describe this phenomenon. Given the number of species shown here to be able to utilize adult carcasses, it seems likely that most, if not all, *Drosophila* species cooperatively digest a variety of substrates. The large salivary glands and clustering behavior of larvae in other dipterans suggest that the larvae of many, or perhaps most, engage in social digestion as well.

Since these results indicate that *Drosophila* larvae are wallowing in a broth of digestive enzymes, including chitinase, an interesting question is how they avoid autodigestion. It also is possible that the behavioral practice of wandering away from the feeding environment just prior to pupation is a means of avoiding digestive attack by other larvae.

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