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Peritrophic membrane role in enhancing digestive efficiency Theoretical and experimental models

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ABSTRACT

The peritrophic membrane (PM) is an anatomical structure surrounding the food bolus in most insects. Rejecting the idea that PM has evolved from coating mucus to play the same protective role as it, novel functions were proposed and experimentally tested. The theoretical principles underlying the digestive enzyme recycling mechanism were described and used to develop an algorithm to calculate enzyme distributions along the midgut and to infer secretory and absorptive sites. The activity of a *Spodoptera frugiperda* microvillar aminopeptidase decreases by 50% if placed in the presence of midgut contents. *S. frugiperda* trypsin preparations placed into dialysis bags in stirred and unstirred media have activities of 210 and 160%, respectively, over the activities of samples in a test tube. The ectoperitrophic fluid (EF) present in the midgut caeca of *Rhynchosciara americana* may be collected. If the enzymes restricted to this fluid are assayed in the presence of PM contents (PMC) their activities decrease by at least 58%. The lack of PM caused by calcofluor feeding impairs growth due to an increase in the metabolic cost associated with the conversion of food into body mass. This probably results from an increase in digestive enzyme excretion and useless homeostatic attempt to reestablish destroyed midgut gradients. The experimental models support the view that PM enhances digestive efficiency by: (a) prevention of non-specific binding of undigested material onto cell surface; (b) prevention of excretion by allowing enzyme recycling powered by an ectoperitrophic counterflux of fluid; (c) removal from inside PM of the oligomeric molecules that may inhibit the enzymes involved in initial digestion; (d) restriction of oligomer hydrolases to ectoperitrophic space (ECS) to avoid probable partial inhibition by non-dispersed undigested food. Finally, PM functions are discussed regarding insects feeding on any diet.

1. Introduction

The peritrophic membrane (PM) is an anatomical structure surrounding the food bolus in most insects with the remarkable exception of Hemiptera and Thysanoptera that have instead a lipid membrane (the perimicrovillar membrane, Kitajima, 1975; Lane and Harrison, 1979; Silva et al., 2004) ensheathing their midgut microvilli. PM is made of proteins (peritrophins) interlocked with chitin fibrils. This anatomical structure is sometimes also called peritrophic matrix, in spite the fact that matrix in biology does not convey the idea of a sheath and suggests a substance that fills a space, like the mitochondrial matrix. The vast literature available on PM is comprehensively reviewed by Peters (1992), by Jacobs-Lorena and Oo (1996) on hematophagous Diptera and three others reviews that emphasize PM structural aspects and PM roles in midgut epithelium protection (Tellam, 1996; Lehane, 1997; Tellam et al., 1999).

Since the insect midgut epithelium lacks a mucus coating, PM functions were supposed to be analogous to that of the mucus that lubricates the mucosa, protecting it from mechanical damage, and to trap bacteria and parasites. Thus, insects deprived of PM may have the midgut cells damaged by coarse food and may be liable to microorganism invasion in some reported cases (Peters, 1992; Tellam, 1996; Lehane, 1997). Nevertheless, taking into account the theory of evolution, it seems unlikely that the ancestral mucus that is found in most animals is replaced by a complicated multi-molecular structure to realize the same protective function.

According to Terra (2001) ancestral insects had their midgut cells covered with a mucus similar to that found in most animals. Later on, the peritrophins evolved from mucins by acquiring chitin-binding domains. The parallel evolution of chitin secretion by midgut cells permitted the formation of the chitin–protein network characteristic of PM structure (see reviews above). Thus, the specific functions of PM (those not played also by mucus) must

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depend on the fact that PM compartmentalizes the midgut lumen into an endoperitrophic space (EDS) (inside PM) and an ectoperitrophic space (ECS) (space between PM and midgut epithelium). Work done with the larvae of *R. americana* showed the significance and importance of the midgut compartments in regulating the initial, intermediate and final stages of polymer digestion (Terra et al., 1979; Ferreira and Terra, 1984). This prompted a large number of papers aimed to study the compartmentalization of digestive events in model insects pertaining to different insect orders. These studies describing the spatial organization of digestive events and determining the PM permeability were reviewed several times (Terra and Ferreira, 1994, 2003, 2005).

Based on those studies, Terra (2001) proposed that the PM functions distinct from those of the gastrointestinal mucus may be divided into primary and secondary functions. Primary functions are those probably evolved under selective pressures, whereas secondary ones are consequences of the chemical properties of PM components (enzyme immobilization and toxin binding). The primary functions found in all insects are: (a) prevention of non-specific binding of undigested material onto cell surface; (b) prevention of excretion by allowing enzyme recycling powered by an ectoperitrophic counterflux of fluid; (c) removal of oligomeric molecules that may inhibit the enzymes involved in initial digestion from inside PM. Primary functions that are restricted to panorpoid insects (dipterans and lepidopterans) are: (a) restriction of oligomer hydrolases to ectoperitrophic space to avoid probable partial inhibition by polymeric food (because of non-productive binding) and putative non-specific binding by non-dispersed undigested food; (b) restriction of monomer production to cell surface causing increased concentration of the final products close to the carriers responsible for their absorption.

From the PM functions proposed, only the prevention of enzyme excretion has some experimental support. Both *R. americana* and *Musca domestica* present a decreasing trypsin gradient along midgut contents (putatively generated by the recycling mechanism) and excrete less than 15% of midgut luminal trypsin after each gut emptying. When the larvae were fed a diet with excess protein, the trypsin gradient along midgut contents becomes less discernible and trypsin excretion increases to 40%. This is exactly what would be expected if the recycling mechanism existed and an increase in undigested dietary protein prevents trypsin from diffusing into the ectoperitrophic space and moving into anterior midgut by the countercurrent (CC) flux of fluid. Subsequently, dye experiments showed the existence of the appropriate fluid fluxes (Terra and Ferreira, 1994, 2005). More recently, experimental evidence that a recycling mechanism also occurs in Lepidoptera and Coleoptera was described (Peterson et al., 1994; Borhegyi et al., 1999; Ferreira et al., 2002).

This paper was carried out to provide experimental and theoretical support for the proposals of PM function described above. For this, a theoretical model for enzyme recycling was advanced and experimental models were developed to detail the recycling mechanism and to test the other proposals. The results confirmed the PM functions proposed.

2. Materials and methods

2.1. Insects

Stock cultures of the yellow mealworm, *Tenebrio molitor* (Coleoptera), were cultured under natural photoregime conditions on wheat bran at 24–26 °C and a relative humidity of 70–75%. Fully grown larvae (each weighing about 0.12 g), having midguts full of food, of both sexes were used.

R. americana (Diptera: Sciaridae) are continuous feeders usually found under decaying plants in banana orchards near the southeast coast of Brazil. The larvae were a gift from Dr. Roberto V. Santelli (University of São Paulo) and we have used only feeding larvae at the end of the 2nd period of the 4th instar (Terra et al., 1973).

Larvae of *M. domestica* (Diptera, Cyclorrhapha, Muscidae) were reared in a mixture of fermented commercial pig food and rice hull (1:2, v/v) (Targa and Peres, 1979). The larvae used in this study were actively feeding individuals at third instar.

Spodoptera frugiperda (Lepidoptera: Noctuidae) were laboratory reared according to Parra (1986). The larvae were individually contained in glass vials with a diet based on kidney bean (*Phaseolus vulgaris*), wheat germ, yeast, and agar and were maintained under a natural photoregime at 25 °C. Fifth (last) instar larvae of both sexes were used in the experiments.

2.2. Hydrolase assays

Aminopeptidase N was assayed in 100 mM Tris–HCl buffer (pH 7.5) for *S. frugiperda* samples and 100 mM phosphate–sodium buffer (pH 8) for *R. americana* samples, using as substrate 1 mM L-Leu-*p*-nitroanilide and following the release of *p*-nitroaniline according to Erlanger et al. (1961). *Aminopeptidase A* was determined with 0.25 mM Asp-β-naphthylamide as substrate (according to Hopsu et al., 1966) in 100 mM Tris–HCl buffer pH 7.5. *Amylase* activity was measured by determining the appearance of reducing groups (Noelting and Bernfeld, 1948) from 0.5% soluble starch in media containing 10 mM NaCl in 100 mM citrate–sodium phosphate buffer pH 5.0 (*M. domestica*), pH 6.5 (*T. molitor*) or 100 mM glycine–NaOH buffer pH 9.5 (*S. frugiperda*). β-*N*-*Acetylglucosaminidase* was determined by following the increase of *p*-nitrophenolate (according to Terra et al., 1979) produced from 1.25 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 50 mM citrate–sodium phosphate buffer pH 6.0. *Carboxypeptidase A* activity was measured with carbobenzoxy-glycyl-L-phenylalanine as substrate in 100 mM Tris–HCl buffer pH 8 and accompanying the increase of Phe (Nicholson and Kim, 1975). *Chymotrypsin* was assayed with 1 μM *N*-succinyl-Ala-Ala-Phe 7-amido-4 methyl-coumarin in 100 mM Tris–HCl buffer (pH 8.5). The substrate is dissolved in dimethyl sulfoxide and then diluted 100 times with buffer. The reaction is stopped with 30% acetic acid and the fluorescence was detected in fluorimeter, with excitation at 380 nm and detection at 460 nm (Alves et al., 1996). In samples containing calcofluor, chymotrypsin was assayed with 0.5 mM succinyl-Ala-Ala-Phe-*p*-nitroanilide in 100 mM Tris–HCl buffer (pH 8.5). The reaction was stopped as before and the absorbance was determined at 410 nm. *Dipeptidase* and *maltase* were assayed with 5 mM Gly-Leu (buffer: 100 mM Tris–HCl pH 8) and 7mM maltose (buffer: 50 mM citrate–sodium phosphate pH 5) as substrate and determining the appearance of leucine (Nicholson and Kim, 1975) and glucose (Dahlqvist, 1968), respectively. β-*Glucosidase* was measured by following the increase of glucose (Dahlqvist, 1968) from 0.15 mM amygdalin in 100 mM citrate–sodium phosphate buffer pH 5.5. At this condition, the measured β-glucosidase activity corresponds to the one that was immunocytolocalized in *T. molitor* midgut (Ferreira et al., 2002). *Trypsin* was assayed with 10 μM carbobenzoxy-Arg-7-amido-4 methyl coumarin in 100 mM Tris–HCl buffer pH 8.0 (*T. molitor*), pH 9.0 (*M. domestica*) or pH 7.5 (*S. frugiperda*). The previous preparation of substrate and details of detection were similar to those of chymotrypsin described above. Trypsin was also assayed with α-*N*-benzoyl DL-Arg-*p*-nitroanilide. Other conditions as before.

All assays were performed at 30 °C in media of the indicated pH values and incubations have been carried out for at least four different periods of time and the initial rates of hydrolysis have

been calculated. Controls without enzyme and others without substrate were included. One Unit (U) is defined as the amount that hydrolyses 1 μmol of substrate (or bond) per min. Enzyme activities were expressed in milli units (mU).

2.3. Distribution of digestive enzymes along the midgut contents

Larvae of *T. molitor*, *S. frugiperda*, and *M. domestica* maintained in their raising diets (diet B) were immobilized by placing them on ice, after which they were either immediately dissected in cold 342 or 125 mM NaCl (*T. molitor* and *S. frugiperda*, respectively) or were previously rinsed with water and blotted with filter paper (*M. domestica*) before dissection in cold 100 mM NaCl. PM with its contents from *S. frugiperda* and *T. molitor* (which have cylindrical midguts) were separated from the midgut tissue and frozen before being divided into eight parts of equal length (numbered from the proximal end). The *M. domestica* midgut is macroscopically differentiated along its extension and its small diameter makes it difficult to recover its contents (see Terra et al., 1988, for details of its morphology). Because of that, *M. domestica* whole midguts, instead of midgut contents, were divided as follows: anterior midgut (divided into three parts: 1, 2, and 3), middle midgut (two parts: 4 and 5) and posterior midgut (four parts: 6, 7, 8, and 9). The nine parts of *M. domestica* midgut were also numbered in sequence from the proximal end. PM and contents (*S. frugiperda* and *T. molitor*) and midgut sections (*M. domestica*) were homogenized in 0.5 mL of double distilled water with the aid of a microhomogenizer (Motor Cordoless, Sigma) and centrifuged at 10,000 × *g* at 4 °C. The resulting supernatants were stored at −20 °C until used. No enzyme inactivation was detected upon storage.

The distribution of digestive enzymes along the midgut of *M. domestica* larvae was also studied in the presence of excess starch and protein. For this, larvae were removed from their raising diets, rinsed with distilled water and, after blotting in filter paper, were placed on layers (2 mL) of 10% (w/v) starch gels (starch diets, diet C) or 4% polyacrylamide gels (prepared as the stacking gel for polyacrylamide gel electrophoresis, Davis, 1964), containing 10% gelatin (protein diets, diet A). After 4.5 h, the larvae were dissected as described above.

2.4. Effect of dialysis on the proteolytic activity of PM contents

Three groups of 40 *S. frugiperda* larvae were dissected and their PM contents (PMC) isolated and homogenized in water (10 mL) to a concentration of 4 animals mL^{-1}. Experimental tubes were prepared by combining 400 μL of the PM contents preparation with 400 μL of 100 mM Tris–HCl buffer pH 7.5, containing 2.5% (1.25% final concentration) azocasein as substrate. The mixtures were placed into dialysis bags (cut off limit, 12 kDa) and submitted to dialysis with stirring against 250 vol. of 100 mM Tris–HCl buffer pH 7.5. Azocasein was chosen as substrate because unless digested it does not pass through the dialysis bag. After 30, 60, 90, 140, and 240 min one dialysis bag of each original sample was removed from the dialysis bath. Following heating the bags at 95 °C for 10 min to denaturate the enzymes, the bags were dialysed for 3 h at 25 °C against the same buffer used previously. A sample of each bag and the corresponding dialysis bath were taken and added to half volume of 50% trichloroacetic acid (TCA). After centrifuging for 5 min at 16,000 × *g* at 4 °C, the supernatants were added to 3 vol. of 2 M NaOH and absorbances read at 420 nm to determine enzyme products. The enzyme activity was calculated taking into account the products recovered from the dialysis bag and bath.

A second experiment was performed as described above, except that the mixture inside the bags was dialysed against buffer without stirring. After enzyme denaturation at 95 °C as described above, the samples were dialysed for another 3 h with regular stirring.

In a third experiment, the dialysis bags containing the mixture were not submitted to dialysis, instead they stood in centrifuge tubes for the same time the others were in dialysis bags, but, subsequently, the samples were treated as before.

2.5. Effect of PM contents on enzymes restricted to the ectoperitrophic space

One hundred *R. americana* larvae were dissected as described in Section 2.3. The ectoperitrophic fluid (EF) was collected by puncturing the large midgut caeca (Terra et al., 1979) and the PM contents was homogenized in water with the aid of a Potter-Elvehjem homogenizer and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatants were filtered by centrifugation (14,000 × *g*, 20 min, 4 °C) through a 100 kDa cut off filter (Centriplus YM, Amicon). The filtrate containing particles smaller than 100 kDa was named PMC <100 and the fraction retained in the filter was labelled PMC >100.

The *R. americana* ectoperitrophic fluid was incubated with the three different PM fractions (total PMC, PMC <100, PMC> 100) for 10 min and the enzymes aminopeptidase, *N*-acetylglucosaminidase, and carboxypeptidase, which are enzymes restricted to the ectoperitrophic space (Terra et al., 1979; Terra and Ferreira, 1983; Ferreira and Terra, 1984), were then assayed. As control, the enzymes were assayed in the ectoperitrophic fluid sample and in non-fractionated PM contents.

2.6. Effect of PM contents on the activity of midgut microvillar enzymes

Three groups of 35 *S. frugiperda* larvae were dissected and their midguts were separated into PM contents and tissue. PM contents were homogenized with water using a homogenizer with a blade rotator (Omni Mixer, Omni, USA) to result in a concentration of 3 animals mL^{-1}, which resemble *in vivo* concentrations.

Midgut tissue, after being rinsed thoroughly with Tris–mannitol buffer (214 mM mannitol, 5 mM EDTA, 5 mM Tris–HCl buffer pH 7.1), was homogenized in the same buffer with the aid of a Potter-Elvehjem homogenizer. The homogenates were then passed through a piece of nylon mesh of 100 μm pore. Filtrates were centrifuged at 5000 × *g* for 10 min at 4 °C to remove intact cells, micro tracheas and other large debris. The pellets (microvillar-size) were diluted in the same buffer and used as a source of microvillar enzymes (aminopeptidase N, carboxypeptidase, and dipeptidase; Ferreira et al., 1994). Maltase is a glycocalyx-associated enzyme (Ferreira et al., 1994) and was also assayed as a control. Three different samples containing 3 or 4 animals mL^{-1} each were used, depending on the experiment. The preparations were not frozen, to avoid freeing the glycocalyx-associated enzymes during the preparation of the microvilli-enriched preparations.

In the experiments, the microvillar-enriched preparation alone or in the presence of different dilutions of PM contents was assayed for microvillar enzymes.

2.7. Digestibility and utilization of food by control and calcofluor-treated larvae

Rates (mg dry wt day^{-1}) and efficiencies (percentages) of food consumption and utilization were defined and calculated according to Slansky and Scriber (1985) as follows: Growth rate (GR) = BT^{-1}, approximate digestibility (AD) = $(I-F)/I$, and efficiency of conversion of digested food (ECD) = $B(I-F)$, where I is food

ingested; F is food excreted as faeces; B is food converted into biomass; T is time in days. The relation $(I-F)/I$ for a particular nutrient gives its digestibility and is expressed as percentage.

Two groups of 12 *S. frugiperda* larvae, each weighing about 340 mg (fresh-weight), were separated. One group was fed with the control diet and the other with calcofluor-containing diet (1% calcolfluor in the standard diet) for 20 h. This time in the presence of calcofluor is enough to completely disrupt PM (Bolognesi et al., 2001). The larvae were placed in individual tubes. The dry-weights of larvae and food (calculated from their fresh-weights with the aid of samples dried at 150 °C for 24 h) were used in calculating the nutritional parameters.

Starch consumed and excreted by control and experimental larvae was determined according to Ferreira et al. (1992). Briefly, faeces and food samples were homogenized in 80 °C ethanol containing 2 mM EDTA, pH 7, boiled for 5 min and centrifuged again at 10,000 × *g* for 10 min at 18 °C. The supernatants were used for starch determinations with the amyloglucosidase procedure of Bruss and Black (1978).

3. Results and discussion

3.1. PM role in enzyme recycling and in decreasing digestive enzyme excretion

A mathematical model was developed (detailed below) that is able to calculate the distribution of digestive enzymes along the midgut contents, given the site of water secretion and absorption or to identify accurately the enzyme secretory site, given the other variables. This model, as any scientific theory (Popper, 1959), was validated by the fact it calculates correctly one set of data out of another. As a corollary, the assumptions used in the development of a model are as reliable as the model itself. This model is useful in functional studies of midgut regions, as it provides an easy way to infer secretory and absorptive sites from enzyme gradients along the midgut contents. Furthermore, attempts to develop models (even approximate) that describe empirically observed processes are important in science, because they usually identify the physical chemical determinants of the processes

The data on any insect are not sufficient to develop a rigorous model of the recycling of midgut digestive enzymes. Nevertheless, the data on *M. domestica* may support an approximate model of digestive enzyme recycling thought to occur in the larval posterior midgut. Based on dye experiments, it was shown that the distal section of the posterior midgut secretes 3.9 μL of fluid during the transit of food along it, whereas 2.3 μL is absorbed in the last section of middle midgut (Espinoza-Fuentes and Terra, 1987). Thus, 1.6 μL hydrate the food (like 1.5 μL that is supposed to hydrate the food in anterior midgut, Espinoza-Fuentes and Terra, 1987) and 2.3 μL pass forward in the ectoperitrophic space and correspond to the countercurrent volume. This is the volume of fluid that moves in ECS during the passage of food along the posterior midgut. The calculated CC volume corresponds to about 40% of posterior midgut endoperitrophic space, which is 6.1 μL (Espinoza-Fuentes and Terra, 1987). It is not known the volume of ECS that may be approximately calculated from the distance between tissue and PM seen in traverse midgut sections. It is assumed here that ECS is 20% of that of EDS.

Table 1 (Model A) shows the distribution of enzyme activity along *M. domestica* posterior midgut (cycle 8) calculated as following. Cycle 0 corresponds to the situation before CC flux started and shows the distribution of the enzyme activity that was secreted in section 1 and was distributed among the other sections. Taking the volume of the ECS as being 20% of that of EDS, the enzyme activity in section 4 of the first cycle will be reduced from 25 to 20 as EDS attains equilibrium with ECS and the ectoperitrophic fluid moved forward adding 5 to section 3 before this section attains equilibrium, resulting in a total of 30. After equilibrium, 24 remains in section 3 and 6 are passed forward. After a new equilibrium, 24.8 remains in 2 and 6.2 are added to 25 in section 1. Note that the activity in section 1 is the sum of the previous activity with those coming from the previous section, as the ECS volume is considered to go to zero in this absorbing section. The same reasoning should be used to calculate enzyme activity in other sections and after new cycles. The volume in equilibrium with EDS in each section is 5% of total EDS volume ($25 \times 20/100 = 5$, where 25 corresponds to the fraction of EDS volume in one section and 20% is the fraction of total ECS relative to total EDS volume). As the CC volume passing by each section corresponds to 40% of EDS (see above), eight cycles (40/5) are necessary to pass all CC volume through the ECS in equilibrium with EDS in a section. In other words, since only 5% of EDS volume is in equilibrium with ECS during each cycle, it is necessary that eight cycles occur in order to attain equilibrium in all 40%.

The described model assumes that CC flux and EDS enter in equilibrium. The time to a solute attains equilibrium between ECS and EDS may be calculated by the Einstein relationship:

$$t = \frac{x^2}{2D}$$

where t is time in seconds, x is the distance in cm traveled by the molecule and D is the diffusion coefficient (Van Holde, 1971). D is 10^{-5} cm^2 s^{-1} (20 °C) for salts and 5×10^{-7} cm^2 s^{-1} for molecules around 50 kDa. The PM pores are around 7.5–9 nm that means that protein molecules of 50 kDa may traverse freely the PM (Terra, 2001 and Section 3.3 below).

The diameter of the midgut of *M. domestica* is 0.005 mm, what means it has 0.0025 cm of radius, which corresponds to the distance a molecule has to travel from the center of the midgut to its border. The time to travel this distance by an enzyme of 50 kDa is 6.25 s. Thus, if the ECS fluid remains in contact through PM with EDS contents for at least this time, both compartments will be in equilibrium.

According to Model A (and also Model B and C), CC fluid remains in contact (through PM) with EDS in each section for about 15 min (emptying time of posterior midgut, 60 min, divided by 4), which exceeds largely the equilibrium time. If the diameter of the midgut is 10-fold larger than that of *M. domestica*, i.e., 0.05 mm, the equilibrium time would increase to 10 min. Thus insects with midgut diameters in excess of 0.05 mm would not attain equilibrium between a CC fluid and EDS.

After several simulations to find a less tiresome way of calculating enzyme distribution data than that of Model A, we came to Model B (Table 1). Model B assumes that the ECS volume is identical of EDS, resulting in the need to calculate only two cycles in the case of the CC volume be 50% of EDS volume. Although this is an arbitrary model, results obtained with Model B are essentially the same as those obtained with Model A. Because of that, only the last simplified Model was used for other conditions with *M. domestica* and other insects for which no data exist regarding CC fluid volume. The main justification for doing this in the last case is the reasonable agreement between theoretical predictions and experimental data to be shown below. Actually, the described model of calculating enzyme distribution data is an algorithm rather than a rigorous theoretically-supported procedure.

Table 1 shows that the experimental distribution of amylase along the posterior midgut of *M. domestica* is quite well predicted by Model A and B, if diet A (protein diet, no starch) was used, whereas in case of diet B (raising diet, with starch) the decreasing gradient is smoother. This would be anticipated, because the

Table 1
Enzyme recycling models (four sections)

Section	Model A			Model B					Model C				
	Cycles			Cycles			Amylase		Cycles			Trypsin	
	0	1	8	0	1	2	Diet A	Diet B	0	1	2	Diet B	Diet C
1	25	31.2	68.3	25	46.9	64.2	61.8	41.1	0	18.8	40.7	38.6	36.2
2	25	24.8	17	25	21.9	17.3	23.6	32.1	0	18.8	21.9	41	34
3	25	24	10.8	25	18.8	12.6	10.9	19.6	50	37.5	25	18.1	25.5
4	25	20	3.9	25	12.5	6.3	3.6	7.1	50	25	12.5	2.4	4.3

Figure corresponds to the distribution of enzyme in the endoperitrophic fluid along the midgut.
Absorption and secretion of water are assumed to occur in section 1 and 4, respectively. The site of enzyme secretion depends on the model: A and B, section 1; C, section 3. Cycle 0 corresponds to the initial condition before starting the countercurrent flux. Cycle 1 (and the subsequent others up till eight for Model A and up till two for Models B and C) refers to the situation after secretion of fluid sufficient to move the fluid in equilibrium in section 4 to section 3 and so on with the fluid originally in section 1 being totally absorbed, with its enzymatic content transferred to the endoperitrophic space. See text for details in calculating data. Amylase and trypsin data come from Fig. 1 and were calculated so that the activity in the posterior midgut added up to 100. Diet A is a protein diet, B is the raising diet, and C is a starch diet.

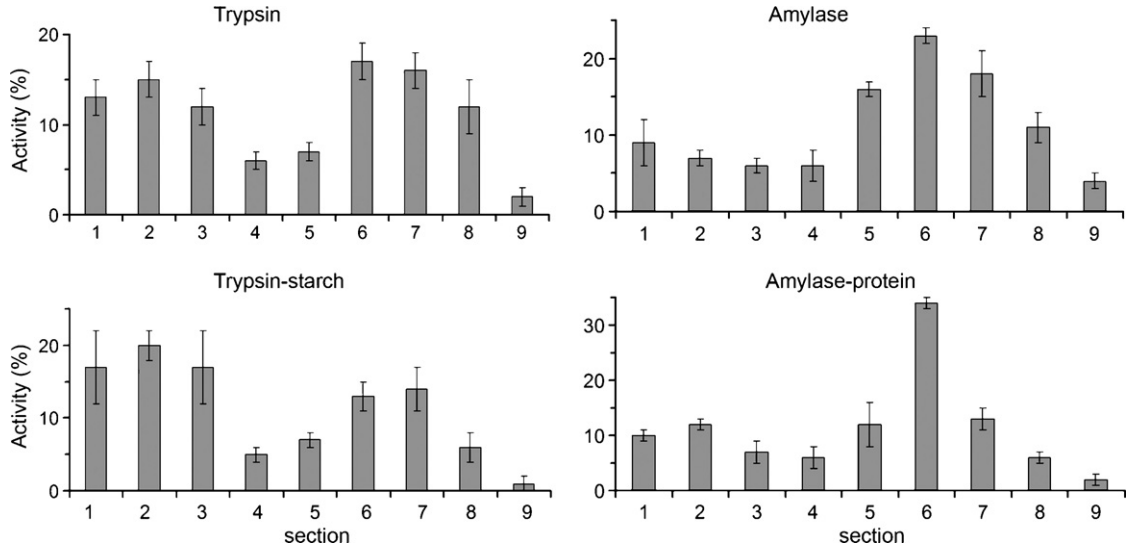

Fig. 1. Distribution of digestive enzymes along the midgut of *M. domestica*. Figures are relative activities (means and S.E.M.) corresponding to determinations performed in three different preparations obtained from 20 larvae each. Trypsin–starch and Amylase–protein correspond to results obtained from larvae ingesting a diet enriched with starch and protein, respectively. Other data were obtained from insects in standard diets.

binding of amylase onto starch would hamper it diffusing across PM. Trypsin distribution is essentially the same in either diet probably because the raising diet (diet B) being poor in protein does not differ significantly from a starch diet (diet C) (Fig. 1).

Either A or B models are not able to predict the distribution of trypsin. Model C, which assumes that trypsin is secreted in section 3, is more successful. Immunocytochemical experiments showed that labeled trypsin is localized along the whole posterior midgut of *M. domestica*, although most of the trypsin was found in cells of sections 1 and 4 (Jordão et al., 1996). The effect of these two major secretory sites on enzyme distribution is apparently that predicted by a single secretion site in section 3. Data on amylase immunocytolocalization is lacking for *M. domestica*.

It is possible to develop models in which there are more than one site for water absorption and/or enzyme secretion. For example, if sections 1 and 2 absorb water similarly, equilibrium in Section 2 is calculated taking into account that ECS was reduced to half of the volume of the ECS from the other sections. Assuming that sections 1 and 2 equally secrete enzyme, in cycle 0, 50% of the enzyme is distributed along all sections and, except for section 1, the same is true for the other 50%. The other calculations should be done as exemplified before.

Table 2 displays four models which predictions can be compared with the experimental results displayed in Fig. 2. To fit the models to the actual enzyme distribution, the sites of enzyme secretion were variously chosen. Model D predicts the distribution of trypsin and chymotrypsin in *S. frugiperda*, Model E of *S. frugiperda* amylase and *T. molitor* β-glycosidase, Model F of *T. molitor* amylase, and Model G of *T. molitor* trypsin. It should be added that dye experiments (Terra et al., 1985) showed that in the

Table 2
Enzyme recycling models (eight sections)

Section	Model D		Model E		Model F		Model G	
	Cycles		Cycles		Cycles		Cycles	
	0	2	0	2	0	2	0	2
1	0	28	0	20.1	0	9.8	0	4.9
2	14.3	13.8	0	11.9	0	6.9	0	3.7
3	14.3	13.4	16.7	15.6	0	10.9	0	6.2
4	14.3	12.8	16.7	14.9	0	16	0	10.2
5	14.3	11.6	16.7	13.6	25	20.3	0	15.6
6	14.3	9.8	16.7	11.5	25	17.2	0	21.9
7	14.3	7.1	16.7	8.4	25	12.5	50	25
8	14.3	3.5	16.7	4.2	25	6.2	50	12.5

Absorption and secretion of water are assumed to occur in section 1 and 8, respectively. The site of enzyme secretion depends on the Model: D, section 2; E, section 3; F, section 5; G, section 7. Only the results of the initial (cycle 0) and final (cycle 2) are shown. Other details in Table 1.

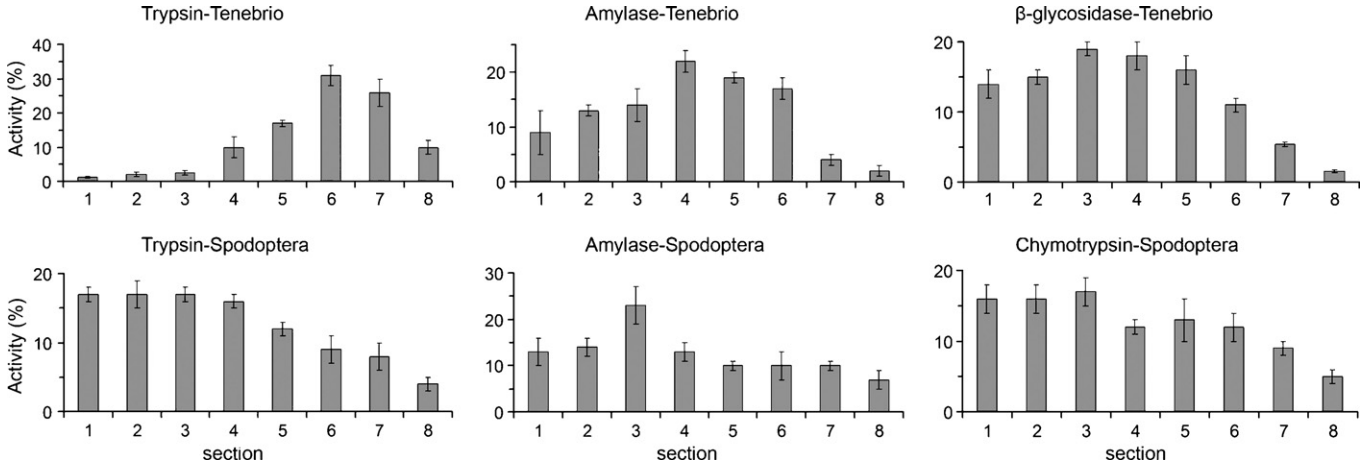

Fig. 2. Distribution of digestive enzymes along the midgut contents of *T. molitor* and *S. frugiperda*. Figures are relative activities (means and S.E.M.) corresponding to determinations performed in three different preparations obtained from 7 (*S. frugiperda*) or 10 (*T. molitor*) larvae each.

T. molitor larvae the anterior midgut absorbs and the posterior midgut secretes water. Although there are no direct data regarding absorbing and secreting water-sites in *S. frugiperda* midguts, it is arguable that they occur in anterior and posterior midgut, respectively, as shown with dye experiments in the larvae of the lepidopteran *Erynnyis ello* (Santos et al., 1986). Some of the theoretical predictions in Table 2 are better than others (compare models with data in Fig. 2). It should be stressed, however, that enzyme binding (unspecific or to the substrate) deviates experimental data from theoretical predictions, as shown before for *M. domestica* amylase. It should also be noticed that the diameter of the midguts of *T. molitor* (1 mm) and *S. frugiperda* (2.5–3.0 mm) precludes the establishment of equilibrium between the CC fluid and EDS. For example, in the case of *S. frugiperda*, taking 2.5 mm as its midgut diameter, the time of a molecule of 50 kDa to diffuse out the PM is 4.3 h, which is approximately the same time the food takes to pass along the whole *S. frugiperda* midgut. Thus, enzyme molecules diffusing across PM cannot come from the middle of EDS, but from its periphery propelled by peristalsis. Peristalsis also would be responsible to move digestive enzymes from the periphery into the core of the food.

There is no immunocytochemical data for *S. frugiperda* chymotrypsin, but the results for trypsin (Jordão et al., 1999) and amylase (Bolognesi et al., 2001) agree with Model D and Model E, respectively, as both describe enzyme secretion in anterior midgut, but are not precise enough to discriminate sections in that region. Immunocytochemical data also agree with the predicted midgut secretion site for *T. molitor* β-glycosidase (Model E) (Ferreira et al., 2002), amylase (Model F) and trypsin (Model G) (Cristofoletti et al., 2001). It is worth noting, however, that Cristofoletti et al. (2001) describes *T. molitor* anterior midgut as the site of amylase secretion and not the middle midgut as predicted by Model F. Nevertheless, no attempt was made by those authors to evaluate the relative contribution of anterior and middle midgut cells in amylase secretion. When this was done for β-glycosidase (Ferreira et al., 2002), middle midgut was found to be more important. We are supposing that the same is true for amylase.

The results discussed above support the view that the theoretical recycling model, and the proposed algorithm, permit (with a good approximation) to compute enzyme activities along the midgut contents, given the water absorption and secretion sites and the enzyme secretory site. The model also predicts, with reasonable approximation, the localization of the enzyme secretory site, given the enzyme distribution along the midgut contents and the sites of water absorption and secretion. Since the absorptive and secretory sites are apparently conserved among the major insect phylogenetic groups (Terra and Ferreira, 1994, 2005), the actual enzyme distribution along midgut contents suffices, as a rule, for predicting the enzyme secretory place. Finally, the assumptions necessary to develop the model (validated by data) indicated that the determinants of enzyme recycling are: (1) the midgut sites of secretion of digestive enzymes and secretion and absorption of fluids; (2) the amount of fluid secreted relative to the size of midgut; (3) diffusion of digestive products that set limits to the midgut diameter in efficient insects lacking peristalsis; (4) residence time of food in midgut; (5) size of enzyme–substrate complexes relative to PM pore sizes that may hamper their diffusion between inner and outer PM compartments.

As a consequence of enzyme recycling, the enzymes are not excreted at the same rate as the food passes through the midgut. The role of PM in decreasing enzyme excretion is clearly shown by the experiment described in Table 3. Insects deprived of PM by calcofluor feeding pass a larger amount of enzymes from the midgut into hindgut. The fraction of a given enzyme that passes into hindgut correspond to the one that escaped to be recovered by the recycling mechanism and therefore is going to be excreted.

3.2. PM role in enhancing polymeric food (initial) digestion

Oligomers (oligopeptides or oligosaccharides) may cause inhibition of initial digestion (the first cleavages of bonds in polymers making them diffusible through a dialysis bag or PM) by product inhibition and (more probable) by serving as substrates of the polymers hydrolases, thus competing with the intact substrate for the enzyme. Product inhibition is a well known phenomenon (Segel, 1975) but we are not aware of studies that look for the effect

Table 3
Enzyme activity (percentage of total midgut activity) in the hindgut of *S. frugiperda* in control and calcofluor-containing diet

Enzyme	Control	Calcofluor
Amylase	6.2 ± 0.2	10.3 ± 0.6
Chymotrypsin	0.20 ± 0.05	0.9 ± 0.4
Trypsin	0.05 ± 0.01	0.9 ± 0.2

Figures are means and S.E.M. based on activity determinations performed in three different preparations obtained from three larvae each. Experimental larvae were fed with 1% calcofluor in the standard diet for 8 h before being dissected. Chymotrypsin and trypsin activities were determined here with colorimetric substrates, because calcofluor fluorescence interferes with measurements carried out with fluorogenic substrates.

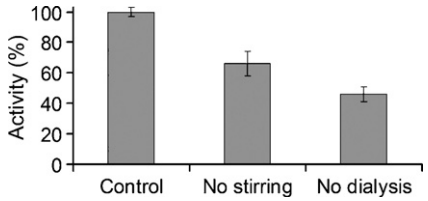

Fig. 3. Proteolytic activity in *S. frugiperda* PM contents at different conditions. Samples dialyzed with stirring (control) and without stirring and in non-dialyzed samples. Figures are percentage of the activity found in control samples and correspond to means and S.E.M. calculated from three samples prepared from 40 *S. frugiperda* larvae each.

of oligomers (serving as substrates) on polymer hydrolysis. Based on this, Terra (2001) hypothesized that an increase in the efficiency of digestion of polymeric food would occur if oligomers were continuously removed from the site of polymer initial hydrolysis (endoperitrophic space) powered by the recycling mechanism associated with the midgut fluxes. Terra (2001) proposed that this role is one of the primary functions of PM in insects of all orders (see review in Section 1).

A model system was used to support this hypothesis. Midgut contents from *S. frugiperda* were placed inside dialysis bags suspended in stirred media. The dialysis bag and stirring were *in vitro* models of PM (with pores) and ectoperitrophic countercurrent flux, respectively. The results (Fig. 3) showed that the proteolytic activity decreases to 77% in bags in unstirred media and to 47% in bags in a centrifuge tube, where no dialysis occurs at all. The possibility that inhibitors of proteolytic activity would be removed on dialysis, thus influencing results, was discounted. Control experiments showed that enzyme activity was not affected by previous dialysis (data not shown). Thus, the data point strongly to the conclusion that PM and the counterflux of fluid in the ectoperitrophic space cause an enhancement of initial digestion by removing competing diffusible oligomers (or inhibitory) products of digestion from the endoperitrophic space.

3.3. PM role in enhancing oligomeric food (intermediate) digestion in Panorpoidea

Terra (2001) advanced the hypothesis that there is an increase in the efficiency of oligomeric food hydrolysis by allowing the transference of oligomeric molecules to the ectoperitrophic space and by restricting oligomer hydrolases to this compartment. In these conditions, oligomer hydrolysis occurs in the absence of probable partial inhibition (because of non-productive binding) by polymeric food and putative non-specific binding by non-dispersed undigested food. This role was considered to be a primary function of PM in panorpoid insects that include dipterans and lepidopterans.

To test this hypothesis, ectoperitrophic fluid from the large midgut caeca of *R. americana* was collected. It was used as a source of aminopeptidase N, *N*-acetylglucosaminidase and carboxypeptidase A, which are enzymes restricted to the ectoperitrophic space, as mentioned in Section 2.6. When those enzymes were put in the presence of PM contents (EF + PMC in Table 4), their activities decrease in relation to controls as follows: aminopeptidase A, 54%; aminopeptidase N, 58%; acetylglucosaminidase, 44%; carboxypeptidase, 8%. These decreases in activity probably result from oligomer hydrolases competitive inhibition by luminal polymers. This is confirmed by the observation that PM contents having particles with less than 100 kDa (PMC <100, Table 4) inhibit less the ectoperitrophic enzymes than those with particles with more than 100 kDa (PMC >100). This condition mirrors *in vivo* situation, as PM pores among larval

Table 4
Effect of PM contents (PMC) on the activity of enzymes restricted to the ectoperitrophic fluid (EF)

Enzyme	EF	EF + PMC <100	EF + PMC >100	EF + PMC
Aminopeptidase A	100	62	77	54
Aminopeptidase N	100	75	58	58
Acetylglucosamimidase	100	37	34	44
Carboxypeptidase	100	38	7	8

Peritrophic membrane contents (PMC) and ectoperitrophic fluid were collected from the midgut caeca of 100 *P. americana* larvae. PMC were fractionated into a sample containing particles with molecular masses smaller (PMC <100 kDa) or larger (PMC >100 kDa) than 100 kDa. Activities in EF were taken as 100 and the activity of each enzyme found adsorbed to PMC was subtracted from those of the EF + PMC (total or fraction) samples. Adsorbed enzymes amount to 27% in the case of carboxypeptidase and to 2–4% regarding the other enzymes in relation to EF activities. The results are means from three determinations in the same sample. Standard errors correspond to 2–7% of the averages.

Diptera vary in the range 7.5–9 nm in diameter according to most determinations (Zhuzhikov, 1964; Terra and Ferreira, 1983; Espinoza-Fuentes et al., 1984; Peters and Wiese, 1986; Miller and Lehane, 1990), which correspond to globular proteins of approximately 100 kDa.

3.4. PM role in enhancing dimeric and small oligomeric food (final) digestion

PM is expected to prevent non-specific binding of undigested material onto midgut cell surface. A consequence of the lack of PM, according to Terra (2001), is the putative impairment of membrane-bound hydrolases (such as aminopeptidase) and carriers of glucose, amino acid and others, leading to a decrease in the efficiency of terminal digestion and absorption. Such hypothetical role was regarded as one of the primary functions of PM to be found in all insects.

To confirm this hypothesis, midgut microvillar-enriched fractions were combined with different dilutions of PM contents and assayed for microvillar enzymes (Fig. 4). The results showed that when the concentration of PM contents approaches that found *in vivo*, the microvillar enzymes aminopeptidase, carboxypeptidase and dipeptidase are clearly inhibited (Fig. 4), whereas the glycocalyx-associated maltase (Ferreira et al., 1994) remains unaffected (Fig. 4). Glycocalyx-associated enzymes are soluble enzymes that are largely trapped in the midgut cell glycocalyx.

3.5. Effect of the lack of PM on nutritional parameters

The experiments described before have shown that in the absence of PM, initial, intermediate, and final digestion should be impaired. As a consequence, in these conditions, nutritional performance is expected to be affected.

Looking for experimental support for that hypothesis, *S. frugiperda* larvae were maintained in a calcofluor-containing diet. Calcofluor inhibits the formation of chitin-containing microfibrils by binding the polysaccharide (Maeda and Ishida, 1967). *S. frugiperda* larvae fed for 8 h with artificial diet containing 1% calcofluor lose their PM (Bolognesi et al., 2001), as previously reported for *Trichoplusia ni* (Wang and Granados, 2000).

AD is maintained constant between calcofluor-treated and control larvae, wherever procedure is used in its determination, whereas CR decreases by 50% (Table 5). Probably the decrease in CR provides more time for food digestion in a less efficient midgut. By the same reasoning, insects lacking the ability to restrict digestive enzymes into the ectoperitrophic space (e.g. Orthopterans and Coleopterans, Terra et al., 1985; Ferreira et al., 1990) should have lower AD or a compensatory lower CR than insects that have that

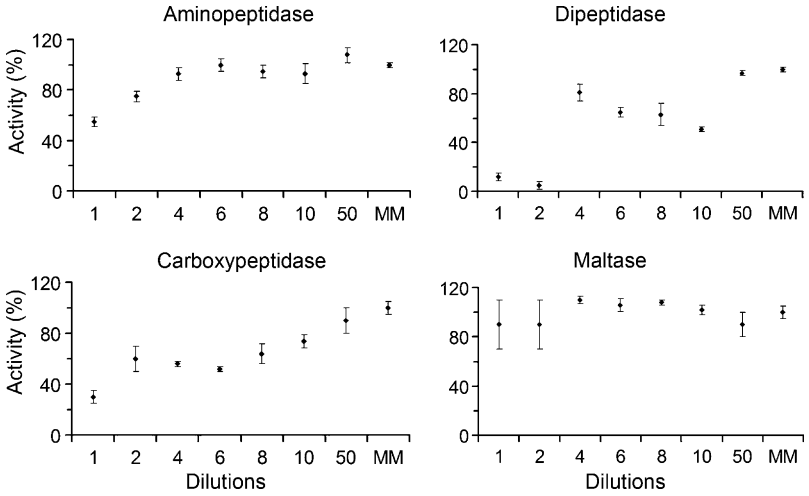

Fig. 4. Activities of midgut microvillar enzymes (MM) from *S. frugiperda* in the presence of midgut contents at different dilutions (1, 2, 4, 8, 10, and 50-fold). Maltase is a glycocalyx-associated enzyme and serves as a control. Activities in MM were taken as 100. Figures are means and range of two independent determinations.

ability (Panorpoidea, like Dipterans and Lepidopterans). In agreement with this, the compilation of data by Slansky and Scriber (1985) is revealing. They used normalized parameters in their comparisons: RCR and RGR (consumption and growth rates by larval mass, respectively). They found the following average parameters. Insects feeding on (a) grass: Orthopterans (AD, 44; RGR, 0.07; RCR, 0.35), lepidopterans (AD, 45; RGR, 0.29; RCR, 1.95); (b) on tree foliage: coleopterans (AD, 50; RGR, 0.03; RCR, 0.30); lepidopterans (AD, 41; RGR, 0.17; RCR, 1.46).

Nevertheless, the five-fold decrease in GR observed in Table 5 cannot be explained by only a two-fold reduction in CR. The GR decrease should be a consequence of the increase in the metabolic costs associated with the conversion of food into body mass rather than of the reduction in CR. The increase in metabolic costs is paralleled by a decrease in ECD, as shown in Table 5.

Data similar to ours were obtained by adding a sub-lethal dose of a chitinase in the diet of *Bombyx mori*. The chitinase increased the permeability of PM and affected the larval nutritional parameters (Rao et al., 2004).

One of the presumed causes of ECD decrease is associated with PM lack or damage that leads to an increase in digestive enzyme excretion (see discussion in Section 3.1). Other probable causes are the impairment of midgut cell surface processes associated with nutrient absorption and the presumed increase in water secretion as a useless homeostatic attempt to reestablish the destroyed midgut gradients.

3.6. PM function in the different insects

The PM as a barrier against invasion by microorganisms has particular relevance in insects that transmit viruses and parasites to human beings, as these microorganisms may have specific developmental phases in insect tissues (Tellam, 1996; Lehane, 1997). Microorganisms invade the insect midgut cells after disrupting the PM with the use of chitinase (Shahabuddin, 1995) or by using a proteinase such as enhancin that affects specifically the peritrophins (Peng et al., 1999; Ivanova et al., 2003). In spite of the importance of PM in human health as a barrier in insect vectors, it is less certain that this PM function is a really major one for the insects themselves (see below).

Table 5
Nutritional parameters determined on *S. frugiperda* larvae fed on control and calcofluor-containing diet for 20 h

Parameters	Control	Calcofluor
AD (%)	50 ± 4	60 ± 6
Starch AD (%)	57 ± 3	63 ± 5
GR (mg day^{-1})	23 ± 4	4 ± 1
CR (mg day^{-1})	105 ± 9	51 ± 4
ECD (%)	44 ± 5	12 ± 4

Figures are means and S.E.M. obtained from 12 larvae in each experimental condition. AD, approximate digestibility; CR, consumption rate; ECD, efficiency of conversion of digested food.

A barrier against microorganism invasion is probably less important for the majority of insects feeding on plants, as exemplified by observations carried out with the moth *T. ni*. Larvae of this insect deprived of PM by calcofluor treatment show high mortality. Examination of dead larvae showed no signs of microbial infection or cell damage by calcofluor, although these larvae were more susceptible to experimental infection (Wang and Granados, 2000). The results may be interpreted as calcolfluor killing larvae by affecting PM functions in digestion.

In the same direction goes the observation that some plants respond to herbivorous insect attack by producing a cysteine proteinase with chitin-binding activity. This proteinase damages the PM, resulting in significant reduction in caterpillar growth caused by impaired nutrient utilization (Pechan et al., 2002; Mohan et al., 2006).

This paper gives experimental support to the previous proposals of PM function that relied mainly on digestive enzyme compartmentalization data. Those functions result in decreasing digestive enzyme excretion and in restricting the intermediate digestion outside PM and the production of the final products of digestion close to their transporters, thus facilitating absorption.

In spite of the reviewed data, there are claims according to which the function of PM in mosquitoes differs from the other insects, particularly from the plant feeding ones. According to these claims, PM in mosquitoes functions as defense against microorganisms (Lehane, 1997), with no role at all in digestion (Villalon et al., 2003). If this was the case, it is not clear why PM is produced only several hours after blood ingestion (Billingsley, 1990), thus leaving the mosquito "defenseless" for such a long time. An alternative explanation for this late PM formation should be that after some hours, digestion undergoing in the blood clot frees oligomers that may impair subsequent digestion, if it is not compartmentalized by PM. This explanation is apparently challenged by the observation that ingested blood protein decreases faster in insects deprived from PM than in control

ones (Villalon et al., 2003). This result means that initial digestion is faster in PM-less mosquitoes, but says nothing regarding intermediate and final digestion and nutrient absorption. Although the number of eggs laid by control and experimental mosquitoes is said to be the same (Villalon et al., 2003), it is by no means certain that the same is true for the number of viable eggs. It should be stressed that small differences in fertility are sufficient to eliminate a group in a population along the time.

As a summary, unless more data are obtained regarding PM function in mosquitoes, the more parsimonious hypothesis of PM function discussed above for other insects should be extended to these organisms.

Acknowledgments

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