COMPARATIVE STUDY OF MIDGUT MORPHOLOGY AND DIGESTIVE PARAMETERS IN WORKERS, QUEENS AND MALES OF *APIS MELLIFERA* L. (HYMENOPTERA, APIDAE, APINAE)[1]

Fabio Cop FERREIRA[2], Carminda da CRUZ-LANDIM.[2]

1 Financial support from FAPESP (Proc. 01/01630-0) and CNPq (Proc.)

[2]Departamentode Biologia, Instituto de Biociências, UNESP, 13506-900 Rio Claro – SP.

ABSTRACT

A colony of eusocial bees is constitute of workers, males and a queen, each one with specific functions in the society. These particular functions imply in differential physiologies and therefore differential nourishment needs. This paper contains an evaluation of the midgut size and histology in different life phases of the mentioned individuals, besides observations about the food intake the food intestinal transit and eletrophoretic proteins pattern of midgut extracts. The results show a parallel between the individual function in the colony and these digestive parameters.

Keywords: individual classes; midgut size, life phases, food intake, intestinal transit.

Correspondence to : Carminda da Cruz Landim
Departamento de Biologia – Instituto de Biociências – UNESP
13506-900 Rio Claro – SP
Telefone : (19)3526-4151
Fax (19) 3526-4136
e-mail: cclandim@rc.unesp.br

INTRODUCTION

The adult components of the eusocial bee colonies have particular social functions that demand different food consumptions. The queen lays most of the eggs that ensure the maintenance of the colony population and eventually provides colony proliferation by swarming. The males have the almost unique function of inseminate the queen during the nuptial flight, while the workers are responsible for every action necessary to maintain the colony and brood growth. The workers tasks are undertaken accordingly to their physiological age and colony needs (16,18,21).

Every of these functions require different energy expenses (9,14,15). This circumstance produces different nutritional requirements among the colony adult individual classes, as well as functions of the same individual along its age. The workers and males feed mainly in honey or nectar and pollen, while the queen eat mostly the royal jelly supplied by the workers. A constant flux of food pass uninterruptedly through the colony components by trophalaxis (6,7,8).

Depending upon the function, the worker needs different rates of protein (21). The newly emerged workers start feeding on pollen (12), because a highly protein nourishment is needed for the complete development of the organism and a little late to produce the royal jelly. On the other hand, the foragers, that flight out the colony, need a more energetic food and prefer nectar or honey (1).

The males, while in the colony , are fed by the nurse worker (13) by trophalaxis, probably with royal jelly, because while young and still immature they, need a highly protein diet . When mature and out of the colony, the males do not feed anymore, once their presence in flowers was only rarely observed (15).

In face of these nutritional differences it may be questioned if the enzymatic content in the midgut and the food transit is the same in all colony individual classes and ages.

The midgut is the main portion of the gut where digestion and absorption takes place. The epithelial cells of its wall synthetizes the digestive enzymes and absorb most of the products of digestion (5,17,22). The eventual digestion occurring in the foregut is due to enzymes from the salivary glands or regurgitated from the midgut, but absorption in this part of the gut never occurs (20). In the hindgut several substances, mainly water and salts, are absorbed and enzymes coming from the midgut may still be active in this gut section.

In spite of be the main digestive portion of the gut, the gross anatomy and histology of the midgut of bees is not differentiated along its length. It is constituted by an epithelial wall, covered outside by two layers of muscles one circular and the other longitudinal (2). Only at the transition between the foregut and the midgut around the stomodeal valve, are a ring of differentiated epithelial cells responsible by the production of part of the peritrophic membrane (11). At the ultrastructural level, Jimenez and Gilliam (17) and Terra & Barber (25) found in epithelial cells of *A. mellifera* the midgut differences that suggest a regionalization of food absorption and enzymes production. According to the authors the enzymes are synthetized in the posterior region of the midgut and the absorption made in the middle of the tube. Nevertheless in *Scaptotrigona postica* Zerbo (personal information) it seems that the synthesis and absorption are made by all principal cells of the midgut, while the water absorption and elimination is made only in determined regions.

In which way the functions performed by the midgut in the food digestion and absorption really induce morphological differentiations among the colony components is another question to be investigated. It is known that the worker honey bee eat pollen during all life cycle and that this food is of difficult digestion and may be of low utilization (9), therefore it is interesting to investigate if there is a delay in the pollen transit through the gut and its regulation.

Therefore the present work was scheduled with the aim of verifiy if there are differences in midgut anatomy and histology, in the quality of intaked food protein content

and food transit and absorption along the midgut that could be related to the special needs of the classes of individuals present in the colony.

MATERIAL AND METHODS

The bees were obtained from the apiary of the Instituto de Biociências (Unesp), Rio Claro, SP., Br. The bees were captured from strong colonies while performing tasks that permited identify their life phase.

The workers were all collected in the same colony and classify as newly emerged (NEW), nurses (NW) and foragers (FW). The queens used were all virgins (VQ) and the males were separated into immature (IM), less than six days old, and mature (MM), more than fifteen days old.

Midgut Measurements

Measurements of the midgut length and width were made in fifteen individuals of each class. In order to avoid the individual size influence, the length of the anterior wing was used as representative of the class size and the data obtained from the measurements submitted to the Spearman correlation test. The indexes between the wing length and length-width of the midgut were treated by the non parametric test of Kruskal-Wallis (ANOVA) at 0,05% significance.

Protein content and eletrophoretic protein pattern

Midguts of ten individuals of each class were dissected in cold buffered saline for insects (7,5% NaCl in phosphate buffer, 0,1M, pH 7,4) and stored at -20 ^{o}C until the used. For the midgut of queens and workers the peritrophic membrane with the food content was

separated from the midgut wall tissues. From the mature males was impossible separate the peritrophic membrane from the gut wall, therefore was used the entire midgut. Extracts were prepared macerating the material with 150µL of distilled water.

The extracts were centrifuged at 10,000 rpm during 5 min. at 5 ^{o}C. The total protein content was carried out according to the method of Sedmark and Grossberg (15) using bovine serum albumin as standard.

The eletrophoretic separation of the protein in the extracts was performed in polyacrylamide gel under deneturated conditions (SDS – PAGE). Aliquots of the extracts, containing 50 µg of protein, were loaded in the gel. The molecular weight standard used were constituted by bovine albumin (MW 66,000), pepsin (MW 34,700), trypsinogen (MW 24,000), β-lactoalbumin (MW 18,400) and lysosim (MW 14,300). The gel was stained with 0,1% Comassie Blue Brillant G-250.

Intestinal Transit

Pollen, candy (a blend of sugar and honey) and 60% sugar syrup were used for study of the food transit.

The queens were not used in this part of the experiment since they don't eat the itens given as food. Fifty individuals of each class for each type of food were separated in groups of 10 in five box of 11 x 11 x 6,5 cm and leave without food for 12 hr, at 32 ^{o}C. After this time they receive the food stained with an atoxic dye, the janus green.

At 6h intervals, samples of five individuals were captured and dissected in order to observe the food transit in the gut. The experiments were performed in triplicate.

Histology

The midguts of three individual of each class were dissected, and each one separated into anterior, median and posterior regions. These regions are separately fixed in

4% paraformaldehyde in 0,1M pH7,5 phosphate buffer containing 0,9% NaCl during 2h at 4 ^{O}C. After dehydration the sample were embedded in JB4 historesin. 5 µm thick sections were stained with hematoxylin and eosin.

RESULTS

Midgut length

The greatest midgut length was found in the males (Fig 1), with averages superior to the other colony individuals. Although the wing length is statistically different in the workers, males and queens (Fig 2), and a positive correlation was verified between the midgut and wing length (Fig 3) only the differences between NEQ and NEW; NEW and FW; NW and FW; NEM and MM are not significant at 5% level (Fig. 1).

The workers present the smallest midguts, but among them the nurses have midgut lengths significantly greater from newly-emerged and forager workers ones. Between the males there are no significant differences, but all newly-emerged classes of individuals were different one from another (Fig. 1).

Protein dosage and electrophoresis

he protein content was greater in the midgut wall extracts them in the peritrophic membrane content (Tables 1A). The greatest value for the midgut wall was found in the newly emerged queen, followed by the forager, nurse and the newly emerged workers in this order. To the peritrophic membrane the higher value was found in the nurse workers and the lower in the newly-emerged ones.

In the whole midgut extracts of males, the values found for the mature ones were almost twice the rates found for the newly-emerged (Table 1B).

The bands pattern of the midgut and peritrophic membrane extracts of newly-emerged queen and worker is very similar, except for the bands of 10.5 kDa present only in queen peritrophic membrane and of 14.3 kDa observed in the midgut wall from newly-emerged workers only (Table 2A).

The nurse workers presented the greatest variety of bands in the wall extracts, while the greatest variety in the extracts showed up in the mature one, wich several bands ranging between 14.3 and 116.6 kDa (Table 2A). The males share the bands of 18.4 and 14.3 kDa (Table 2B).

Intestinal Transit

The movement of the food along the gut and its absorption was observed throughout the localization of the stained food into the lumen and by the staining of the gut wall, indicative of absorption.

The transit of syrup and candy through the gut was faster than the pollen in all cases, but the ingestion of food and it dislocation and absorption varied among the individual classes (Table 3). The syrup and candy were taken in great amounts. In individuals fed with candy and syrup the crop appeared always loaded, but in that fed with pollen, even in nurse workers they frequently were empty or with few grain. Frequently, after 10 hr the stained pollen was yet not present in the hindgut (Table 4).

No stained pollen was found in the gut of newly-emerged or mature males during the time they stay in the cages (Table 4). The transit of the syrup and candy was more rapid in mature male guts . In these individuals the syrup was seen in the ileum 2.5 hr after the feeding beginning and the candy just after the first hour (Table 3). Males fed with candy presented absorption in the posterior portion of the midgut and in the ileum as could be detected by the reddish color of these portions of the gut wall. The food residues in the

rectum lumen change the original green color to a reddish one, perhaps due to differences of pH between the midgut and hindgut.

Although the newly-emerged workers feed in the disponible food in their cages, after the 20 hrs of caging the food was not yet seen in the hindgut, neither absorption could be detected. In the nurse worker, both syrup and candy pass through the gut with similar velocity, and 6 hrs after caging was found in the ileum (Table 3). This kind of worker, when fed with candy presented reddish color in the midgut wall after 2.5 hrs and later also in the ileum wall. Forager workers fed with syrup showed similar results to the nurses. In both cases the food passed rapidly through the midgut. The food was detected in the ileum 4 hrs after caging and in the rectum 6 hrs after (Table 3). A reddish color was observed in the midgut and ileum wall of this class of the worker.

In spite of the individuals have been maintained caged without food during 12 hrs, some of the nurse and forager workers, collected 1 hr after feeding still presented not stained pollen in the midgut and rectum. The newly-emerged workers and males even before starting feeding presented some material in the midgut, perhaps cellular residues left from metamorphosis. In the mature males the gut was totally empty before the food is given.

Histology

Some differences could be detected in the midgut epithelium and content. The newly-emerged queen gut presented no food at all, although several layers of the peritrophic membrane may be observed, mainly in its anterior region (Fig 4A). In the median and posterior regions of the gut the peritrophic membrane layered condition is not well discernible and the membrane appear as a thick layer of amorphous material (Fig. 4B, C). In the median region cellular debris are present between the epithelium and the

membrane (Fig 5B). The epithelial folds are more accentuated in the anterior region and practically absent in the posterior midgut (Fig. 4C).

The newly-emerged male has no food in the midgut, but show a well structured peritrophic membrane in several layers, mainly in the anterior region (Fig. 5A). As in the queen this arrangement tend to change in the median and posterior regions, with loss of the layered feature of the membrane (Figs. 5B, C). The folds of the epithelium are present along all midgut length. The cells seem to produce an amorphous material strongly basophilic which can be seen between the peritrophic membrane and the lumen and between the membrane and the epithelium (Fig. 5A, B). In the median region this material acquires a powder aspect (Fig. 5B). The mature males do not have a structured peritrophic membrane. The epithelial folds are present along all midgut, but the epithelial cells seem damaged, presenting very small picnotic nuclei (Figs. 5D-F). The midgut lumen has no food, but cellular debris (Figs 5E, F).

The peritrophic membrane of the newly-emerged workers are less structured than the ones of queen and males, as in any region it shows no organized layered structure (Fig. 6A-7). In the anterior region, as in the males a strongly basophilic material seems to be detaching from the epithelium that may be peritrophic membrane constituent (Fig. 6A). In the middle and posterior region this is less evident (Figs 6B, C). The epithelial folds almost disappear in the posterior region and many bubbles are observed detaching from the cell apices (Fig. 6C).

Pollen was never seen in sections of newly-emerged individual despite of the class, showing that they have not fed yet. In the nurse and forager workers pollen was present. The peritrophic membrane of the newly-emerged individuals appear thicker, probably due to the small amount of food in the lumen (Figs. 6D-F). Also the layers become less evident as the individuals get older (Figs. 6G-I).

Layered peritrophic membrane is not seen in the posterior region of nurse workers gut, probably, due to the great amount of pollen into it (Fig. 6F).

Nests of regenerative cells can be seen along all the midgut of all classes of individuals.

DISCUSSION AND CONCLUSIONS

Several aspects must be considered in the discussion of the present results.

There is a highly positive correlation between the midgut length and the individual size in such a way that eventual differences between life phases of the same class of individuals a must be due to the midgut filling up with food and not to a real difference in the midgut size.

In general the youngest workers and males need more proteic food to physiological maturation of the organs and the queens need proteic food during all life because of the eggs production, but the other classes, when get old, need more energetic than proteic food. The workers start eating pollen just few hours after emergence (9.12) corroborating the need of proteins. Opposite to it the foragers rarely were seen eating pollen. Therefore the longer and wide midgut present in the nurse workers are due to the amount of pollen into it, which promotes its enlargement opening the epithelial folds. The pollen transit through the gut being slow (3) promotes the accumulations that enlarges the organ. The forager worker that feed in nectar or honey, foods of prompt absorption, have shorter midguts, as the food do not accumulates into it (4).

Although some authors (13) report that the young males need highly proteic nourishment, in the present investigation there is no evidences of the males eating, pollen . This is justified as the only function of the males is the queen insemination. When the male emerge the spermatogenesis has already been finished and the sperm is descending from the testes to the seminal vesicles. Therefore even for the gametogenesis, proteins are anymore needed.

Another aspect of the present results is the protein concentration in the extracts. The higher rate seen in the nurse worker peritrophic membrane extracts is due to the pollen into it, and the lower rate in the newly-emerged worker due to the fact that they did not start eating yet. In the midgut wall was assumed that the rate of protein content is due both to the tissue proteins and to variations in their differential synthesis in worker different life phases. In the males where the whole midgut was treated the newly-emerged ones, presented the lower values, probably because the enzymes synthesis was not initiated. The observed difference between newly-emerged and mature males is due to the fact that mature males eat candy only.

The differences between the extracts from the midgut wall and peritrophic membrane are due to structural proteins present in the pollen and in the midgut cells and to stored proteins in both kind of cells. The nurse workers showed the greatest variety of bands in the peritrophic membrane extracts although this was expected in nurses and foragers. Although the newly-emerged worker has not started feeding yet, cells discharged during the midgut wall reorganization are digested in it lumen (2). Zerbo (personal information) found in the midgut of foragers of meliponines only a few grains of pollen suggesting that they do not ingest this kind of food. This is in accordance with the present observations, as it was observed that this class of physiologies and functions of these individuals.

ACKNOWLEDGMENTS

The authors are indebted with FAPESP and CNPq for the financial support and with the anonymous referees that with their criticism contributed to improve the manuscript.

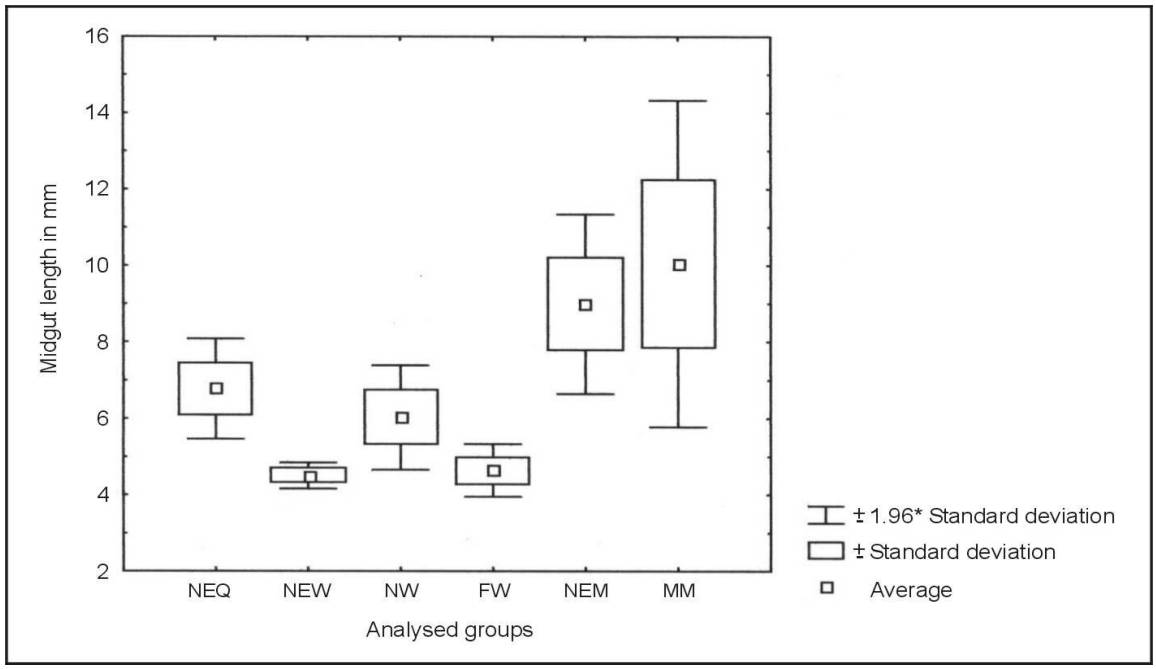

FIGURE 1. Length of the midgut in newly emerged queens (NEQ), newly emerged workers (NEW), nurse workers (NW), forager workers (FW), newly emerged males (NEM) and mature males (MM), of *Apís mellifera.* The differences amongst NeQ and NEW; NEW and FW; NW and FW; NEM and MM are significant at 5%.

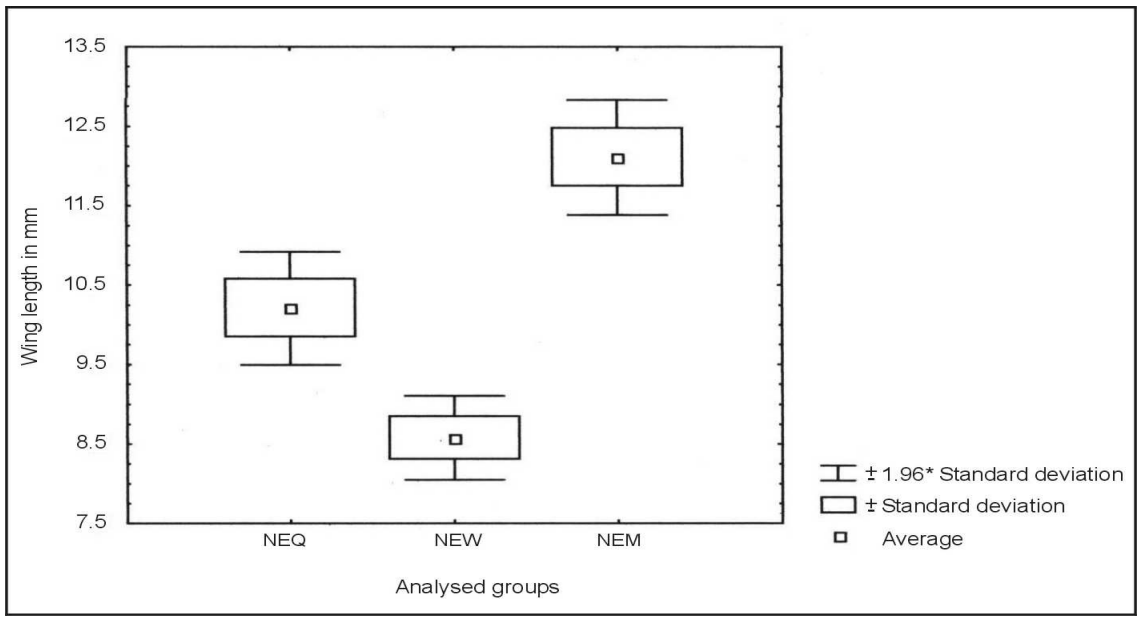

FIGURE 2. Length of the wings of newly emerged queens (NEQ), newly emerged workers (NEW) and newly emerged males (NEM) of *Apis mellifera*.

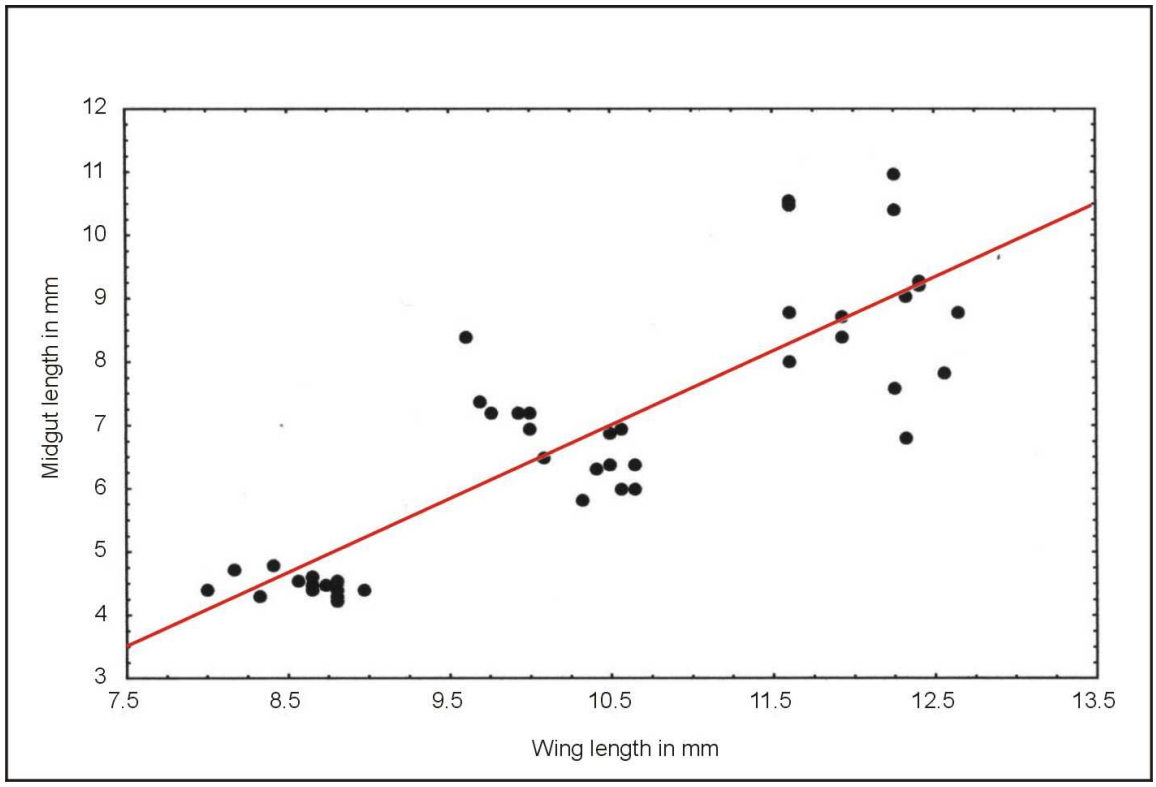

FIGURE 3. Spearman correlation between wing and midgut length in newly emerged individuals $R=0.81$; $p<0.05$.

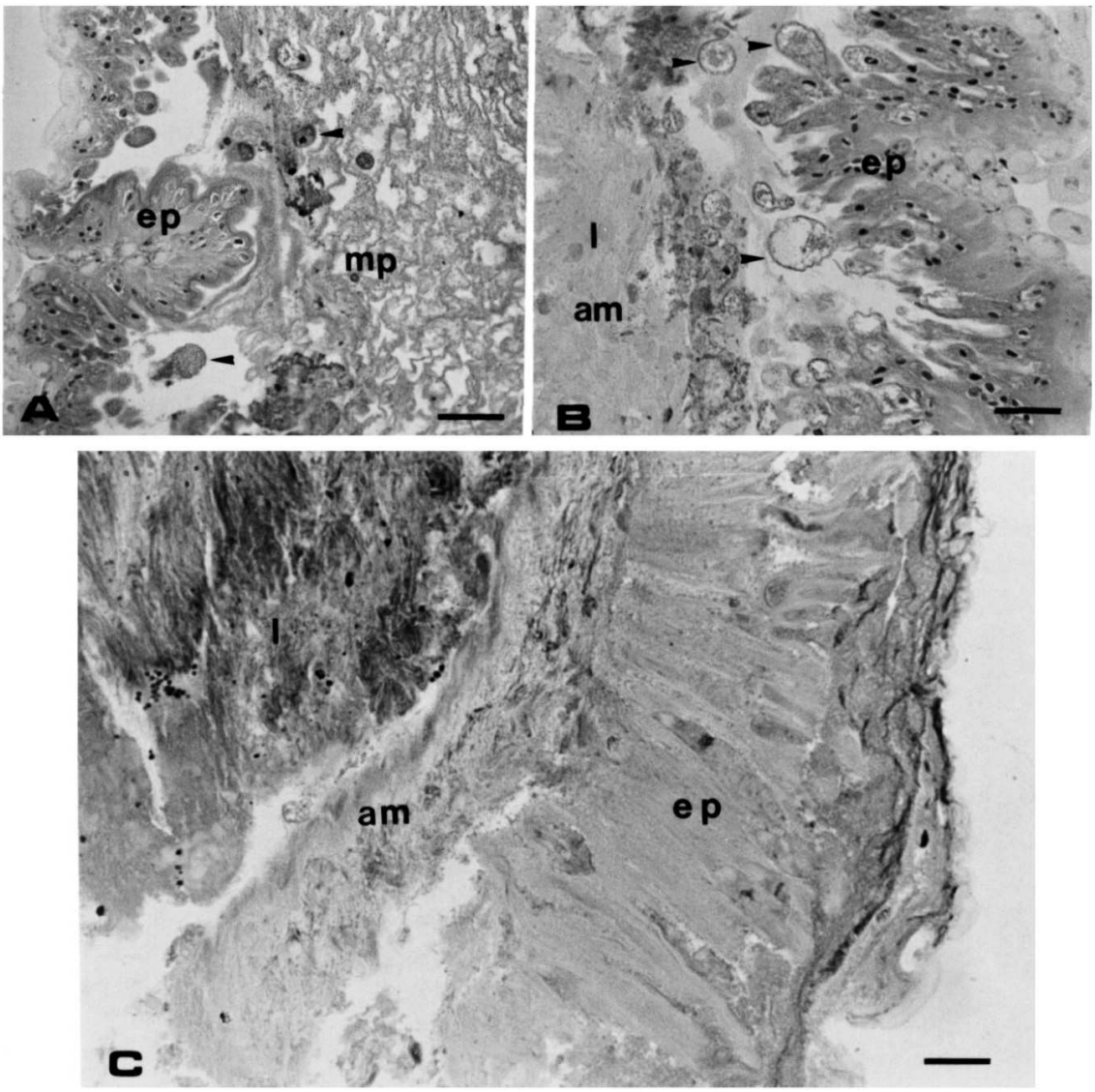

FIGURE 4. Light micrographs of the midgut of newly emerged queen of *A.mellifera*. A. Anterior regions showing the epithelial folds (ep) and the several layers of the peritrophic membrane (mp). Note bubbles (arrow heads) liberation from the epithelial cells apices. B. Median region showing the less compiscuous epithelial folds (ep), the great amount of bubbles apparently empty (arrow heads) and the amorphous peritrophic membrane (am). C. Posterior region without epithelial folds (ep) and desorganized peritrophic membrane (am). Bar=50 mm.

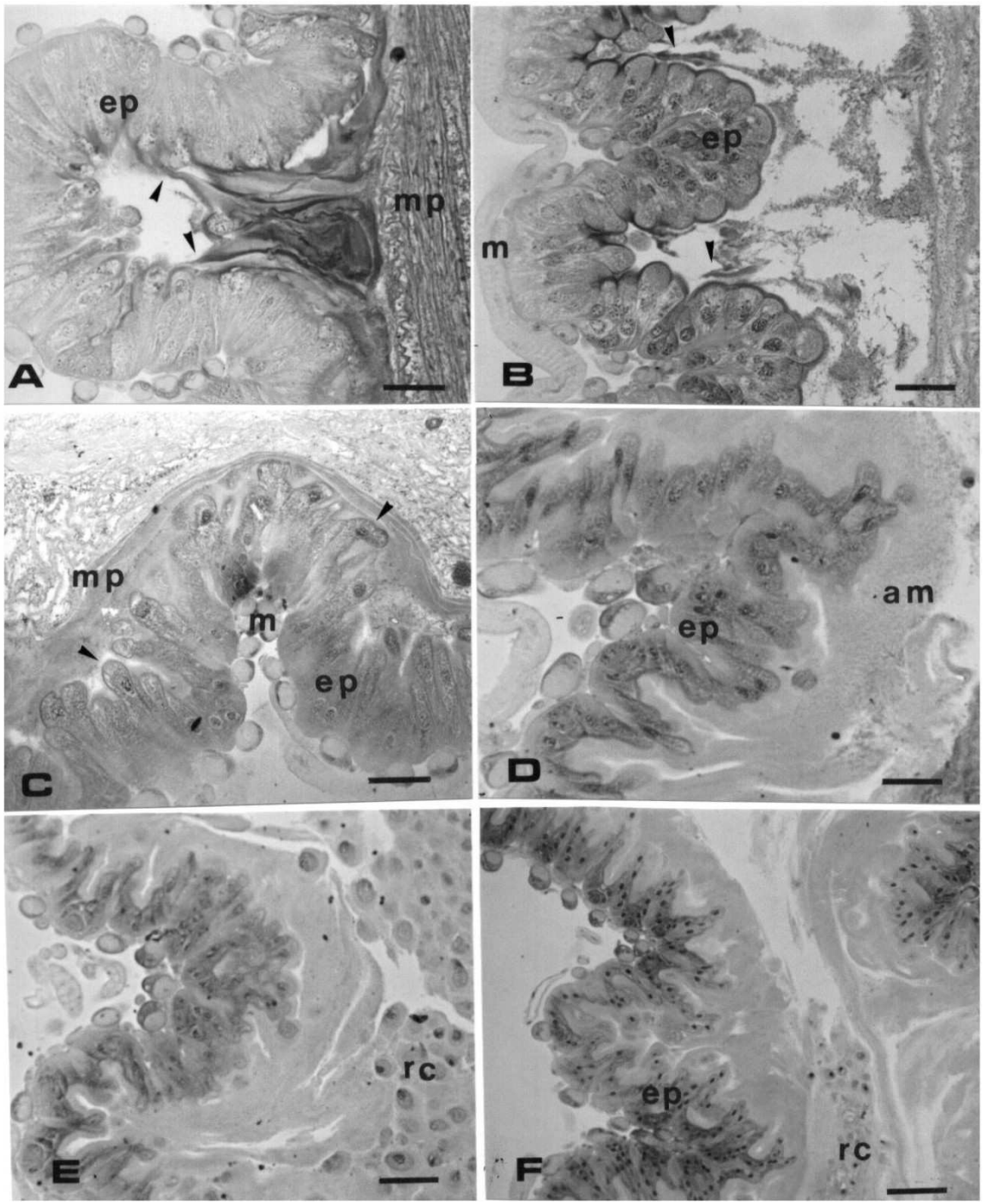

FIGURE 5. Light micrographs of the newly emerged males (A,B,C,) and mature males (D,E,F) of *A.mellifera*. A. Anterior region showing the well structured epithelium (ep) and peritrophic membrane (mp). Note the production of amorphous material (arrow heads) by the epithelial cells. B. Median region with better structured epithelial folds (ep) and less structured peritrophic membrane layers. Note granular material (arrow heads) being liberated by the epithelium. C. Posterior region showing bubbles formation in the epithelium (ep) and less compiscuous folds. D.E.F. Note in the mature male the absence of a structured peritrophic membrane and the slender epithelial cells (ep) showing picnotic

nuclei and in the median and posterior region the midgut lumen full of cellular residues (rc) detached from the epithelium, some containing nucleus. M=muscles; am=amorphous material. Bar=50 mm.

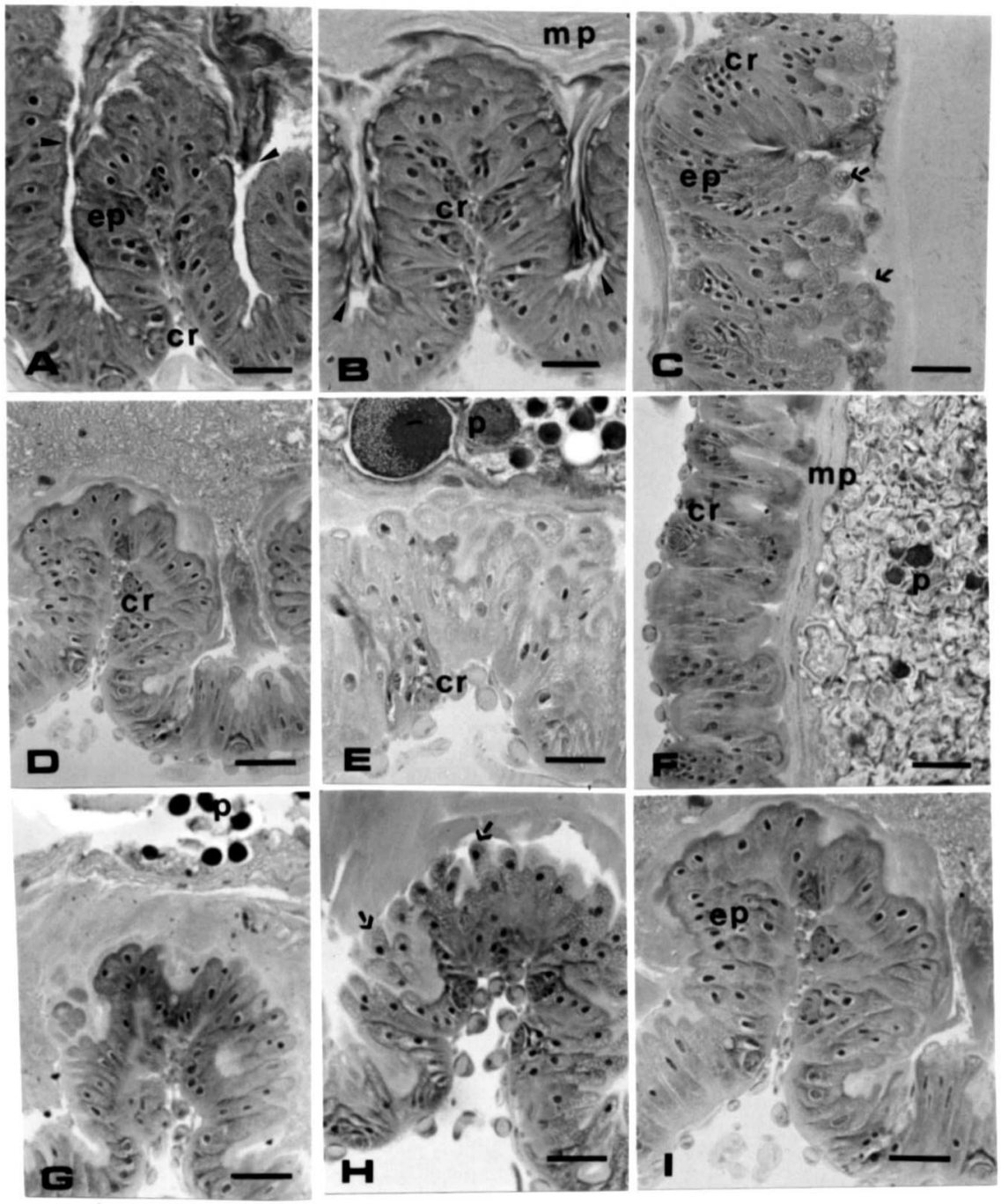

FIGURE 6. Light micrographs of newly emerged workers (A,B,C), nurse workers (D, E,F) and forager worker (G,H,I) of *A.mellifera*. Note in the newly emerged workers the well structured epithelial folds (ep) in the regions anterior (A) and median (B) and the less structured in the posterior (C) where numerous bubbles (arrows) are detaching from the

cells. The peritrophic membrane (mp) as well, is less structured in this region. Note the peritrophic membrane material being produced in A and B (arrow heads). The midgut of the nurse workers maintain the same pattern, but it lumen is full of pollen (p). In the forager workers, the epithelium (ep) show signs of degeneration, the peritrophic membrane is less structured and few pollen grains (p) are present in the lumen (l). In the median region the epithelial cells apices are bulbous (arrows). cr=regenerative cells nests. Bar=50 mm.

TABLE 1A. Average protein content in extracts from the midgut (Mg) wall and peritrophic (Pm) content in newly emerged queens (NEQ), newly emerged workers (NEW), nurse workers (NW) and forager workers (FW) of *Apis mellifera* in □g/□l individual.

	NEQ	NEW	NW	FW
Mg	0.892	0.475	0.592	0.634
Pm	0.431	0.167	0.735	0.282

TABLE 1B. Average of protein content in the midgut (Mg) of newly emerged (NEM) and mature males (MM) of *Apis mellifera* in □g/□l.

	NEM	MM
Mg	0.475	0.892

TABLE 2A. Eletrophoretic pattern of the prtoeins of the midgut wall and peritrophic membrane content in newly emerged queens (NEQ), newly emerged workers (NEW) nurse workers (NW) and forager workers (FW) of *Apis mellifera*. MW=molecular weight.

	Midgut Wall				Peritrophic Membrane			
MW	NEQ	NEW	NW	FW	NEQ	NEW	NW	FW
44,5	Ω				Ω			
43,5			Ω					
34,7						Ω		
31,7			Ω				Ω	
24			Ω			Ω	Ω	
15,4	Ω				Ω	Ω		Ω
14,3	Ω							
10,5		Ω		Ω	Ω	Ω	Ω	Ω

TABLE 2B. Electrophoretic pattern of the proteins of the midgut of newly emerged males (NEM) and mature males (MM) of *Apis mellifera*. MW=molecular weight.

MW	NEM	MM
116.6	Ω	
58	Ω	
33.3	Ω	
24	Ω	
18.4	Ω	Ω
14.3	Ω	Ω

TABLE 3. Intestinal transit of syrup and candy in newly emerged workers (NEW), nurse worker (NW) forager workers (FW), newly emerged males (NEM) and mature males (MM) of *Apis mellifera*. Ingested food located in the anterior (a), median (m), posterior (p), anterior illeum [I(A)], illeum (I) and rectum [R(A)].

	Syrup					Candy				
	NEW	NW	FW	NEM	MM	NEW	NW	FW	NEM	MM
1h	a	m	m	-	p	m	p	m	-	I(A)
2.5h	m	p	p	-	I	m	p(A)	p	-	R(A)
4h	p	p	I	-	I	m	p(A)	I(A)	-	R(A)
5.5h	p	p	I(A)	-	I	m	p(A)	I(A)	-	R(A)
6h	p	I	R(A)	-	I	p	R(A)	R(A)	-	R(A)

TABLE 4. Intestinal transit of pollen in the newly emerged worker (NEW) nurse worker (NW), for forager worker; newly emerged male and mature male of *Apis mellifera*. there are no data a, m, p – respectively – anterior, median and posterior regions of the midgut.

	Pollen	
	NEW	NW
2h	m	a
4h	m	m
6h	p	m
8h	p	m
10h	p	m

REFERENCES

1- Camargo CA (1972) Determinação das castas em *Scaptotrigona postica* Latreille (Hymenoptera:Apidae). *Rev. Bras. Biol.*, 32,133-138.

2- Cavalcante VM, Cruz-Landim C (1999) Types of cells present in the midgut of the insects: a review. *Naturalia*, São Paulo, 24,19-40.

3- Crailsheim K (1998ª'') Regulation of food passage in the intestine of the honey bee (*Apis mellifera*). *J. Insect Physiol*., 34,85-90.

4- Crailsheim K (1988b) Intestinal transport of sugars in the honeybee (*Apis mellifera* L.). *Insect Physiol*., 34,839-845.

5- Crailsheim K, Stolberg E (1989) Influence of diet, age and colony condition upon the intestinal proteolytic activity and size of hypopharyngeal glands in the honey bee (*Apis mellifera* L.). *J. Insect Physiol.*, 35,595-602.

6- Crailsheim K (1990) Protein synthesis in the honey bee (*Apis mellifera* L.) and trophollactic distribution of jelly among imagos in laboratory experiments. *Zool. Jb. Phisiol*., 9,303-312.

7- Crailsheim, K (1991) Interadult feeding of jelly in honey bee (*Apis mellifera* L.) colonies. *J. Comp. Physiol*., 161B,56-60.

8- Crailsheim K (1992).The flow of jelly within a honey bee colony. *J.Comp.Physiol.B.*, 162,681-689.

9- Crailsheim K, Schneider LHW, Hrassnigg N, Bühlmann G, Brosch U. (1992) Pollen consumption and utilization in worker honey bee (*Apis mellifera carnica*): dependence on individual age and function. *J. Insect Physiol*., 38,409-419.

10- Cruz-Landim C, Serrão, JE, Silva-de-Moraes, RLM (1996) Cytoplasmic protrusions from digestive cells of bees. Cytobios, 88,95-104.

11- Cruz- Landim, C (1985) Origin of the peritrophic membrane of the adult *Apis mellifera* L. (Hymenoptera, Apidae). Rev. Bras. Biol., 45, 207-219.

12- Dietz A (1969) Initiation of pollen consumption and pollen movement through the alimentary canal of newly emerged honey-bees. *Ann. Ent. Soc. Am*., 62,43-46.

Recebido em 28.03.2004
Aceito em 13.10.2004