ISSN- 0102-9010

FMRFAMIDE-LIKE IMMUNOREACTIVE MIDGUT ENDOCRINE CELLS IN DIFFERENT CASTES OF THE BEE *Melipona quadrifasciata anthidioides* (APIDAE; MELIPONINI)

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ABSTRACT

FMRFamide-like immunoreactive cells have been identified in almost all insect species studied. However, the functions of this peptide are still unknown, although several studies have suggested that FMRFamide may play a role in controlling peristalsis, digestion, development and reproduction in insects. Differences in the number, morphology, and distribution of FMRFamide-like cells have been observed among insects. Social bees are characterized by the presence of well-defined castes, each with a different behavior, energy demand and nutrient consumption. In this work, we used immunofluorescence to assess the number, morphology, and distribution of FMRFamide-like immunoreactive cells in different castes of the bee *Melipona quadrifasciata anthidioides*. These immunoreactive cells were observed only in the posterior region of the midgut, whereas FMRFamide-immunoreactive nerve fibers were more abundant in the fore- and hind-midgut boundary. However, there were no differences in the number and distribution of FMRFamide-like cells among the castes. This localization of immunoreactivity may indicate that the nervous system controls the passage of food through the cardiac and pyloric valves, while the passage of food through the midgut is controlled by midgut endocrine cells. The number, morphology and distribution of midgut FMRFamide-like cells were not influenced by behavior, feeding habits, caste, or sex in this species.

Key words: FMRFamide, immunofluorescence, midgut, nerve terminals, stingless bee

INTRODUCTION

The gut endocrine cells of insects were first described based on their morphological and ultrastructural characteristics [2,4,9,10,15-17,28,39,42,48,56]. In insects, these cells are not easily identified by the classic light microscopic techniques used to detect them in higher organisms [2,39,32]. However, immunohistochemistry using antibodies raised against endocrine cells in vertebrates have allowed the identification of similar cells in several species of insects [1-4,8,17,25,31,39-42,51,61].

According to Sehnal and Zitnan [51], endocrine cells may play a crucial role in controlling peristalsis, digestion, development and reproduction in insects. Indeed, the content of FMRFamide (Phe-Met-Arg-Phe-amide) in endocrine cells of *Locusta migratoria* is influenced by the alimentary status [35] and by the diet composition [62]. There is also a relationship between the concentration of FMRFamide in hemolymph and neurohemal areas and reproduction in *Rhodnius prolixus* [54].

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Observations in *Hoembia sp.* (Embioptera) have indicated differences in the types of FMRFamide-positive cells in this species [61]. In males, only FMRFamide-positive cells of the closed type were found, whereas in females, open cells were also observed. Differences in the number, type, and distribution of FMRFamide-like immunoreactive endocrine cells have also been observed in *Manduca sexta* larvae infested with the braconid wasp *Cotesia congregata* compared to uninfested larvae [60].

Social bees have well-defined castes, which include a queen bee, responsible for egg laying, worker bees, which do almost all of the other tasks in the colony, and drones, responsible for fertilizing the queen [24,37]. The workers show polyethism, which is determined by physiological factors but can be adapted to the colony's needs. The different tasks done by the workers are executed in a specific sequence and change according to the worker's age or physiological stage. Young workers usually feed the brood while older ones are foragers [24,27,37]. These different tasks require different nutrients. Thus, workers caring for the brood require a high protein diet to produce a hypopharyngeal gland secretion which is

supplied to the larvae and to the queen, while the foragers need greater energy-rich nutrients. Thus, young workers consume more pollen whereas the foragers have a diet rich in honey [19,26,52].

In the Meliponini, the workers feed the males through trophallaxis contact while the latter remain in the nest [12]. After leaving the nest, the males may either collect nectar from flowers or may not eat anymore [33,43,52]. The queen consumes pollen and glandular secretion supplied by young workers [52]. These differences in diet are also reflected in the ability to digest pollen as observed among castes of *Apis mellifera carnica* [57].

FMRFamide and/or FMRFamide-related peptides (FaRPs) are highly conserved in the animal kingdom [36] and are the most prevalent peptides in endocrine cells and nerve terminals of the insect gut [49,61]. FMRFamide-like immunoreactive endocrine cells and neurons have been identified in the midgut of *Melipona quadrifasciata anthidioides* [41].

In this study, we used immunofluorescence to assess whether there were significant differences in the FMRFamide-like peptide immunoreactivity of endocrine cells among different castes of *M. quadrifasciata anthidioides*, and whether such differences could be related to the feeding habits and functions of the castes.

MATERIAL AND METHODS

Animals

Ten specimens of each caste (queens, males, and nurse and forager workers) of *M. quadrifasciata anthidioides* were collected from their nests at the Central Apiary of the Federal University of Viçosa, in the State of Minas Gerais, Brazil. Nurse workers were collected from the brood rearing area of the nest, foragers were obtained from the nest entrance while they were arriving from the field, and males were collected near to the nest entrance. The bees were killed by freezing and their digestive tracts were removed in insect saline solution (0.1 M NaCl, 20 mM KH_2PO_4 and 20 mM Na_2HPO_4) and transferred to picric acid formaldehyde solution [55] for 12-24 h.

Immunofluorescence

After fixation, the midguts were isolated and processed using a modification of the procedure described by Franklin and Martin [18] and An *et al.* [1]. Initially, the tissues were washed three times (1 h each) in phosphate-buffered saline (PBS), pH 7.4, containing 1% Triton X 100 (PBST), followed by incubation in 10% normal goat serum in PBST for 2 h at room temperature. After three washes (30 min each) in cold PBST, the tissues were incubated with an anti-FMRFamide antibody (1:500) for 24 h in a humid chamber at 4°C. The tissues were then washed in cold PBST (3x 30 min) with periodic shaking and incubated with secondary antibody (1:400) at 4°C for 24 h. This was followed by further washing (3x 30 min in cold PBST) and incubation with a streptavidin-fluorescein isothiocyanate complex (1:100) for 12 h at 4°C. A final series of washes as described above completed the staining. Some midgut fragments were used for whole mounts, while others were quickly dehydrated in alcohol and embedded in (2-hydroxyethyl)methacrylate (Historesin, Leica).

Serial sections (4 μm thick) were stained for 20 min with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 0.2 mM in PBS; Sigma), then washed in water and mounted under glass coverslips with Vectashield. The fragments used for whole mounts were mounted under coverslips using phosphate-buffered glycerin jelly [5]. The edges of the coverslips were then sealed using nail varnish and analyzed and photographed immediately.

Antisera

The rabbit anti-FMRFamide antibody was obtained from Peninsula Laboratories (San Carlos, CA, USA), the goat anti-rabbit IgG, biotin conjugate was from Sigma (St. Louis, MO, USA) and the SAv-FITC was from Pharmingen (San Diego, CA, USA). The specificity of the primary antibody was checked by replacing the specific antiserum with normal rabbit serum. The stained tissues were observed with an Olympus BX-60 fluorescence microscope fitted with WB, WU, and WG filters. The cells were photographed using Superia ISO 400 films (Fuji).

Cell counting and statistical analysis

The terminal 1 mm of the midguts of three specimens of each caste was used to compare the number of FMRFamide-like immunoreactive midgut endocrine cells among the castes. The number of reactive cells in at least six longitudinal, randomly, chosen sections was counted. All statistical comparisons were done using ANOVA, with a value of $p < 0.05$ indicating significance.

RESULTS

The cells with FMRFamide-like immunoreactivity were concentrated in the posterior area of the midgut in all castes, as previously observed in workers [42], while FMRFamide-like immunoreactive neurons and nerve fibers were more abundant in the anterior midgut and in the terminal portion of the posterior region of the midgut.

Whole mounts were appropriate for observing neurons and nerve fibers (Fig. 1), but were inadequate for observing endocrine cells, perhaps because the gut wall was thicker in this species. In contrast, sections from Historesin-embedded tissue were suitable for observing immunoreactive endocrine cells (Fig. 2) and nerve cells (Fig. 9) and fibers (Fig. 10).

Despite of the different functions of each caste in the colony, no significant differences were detected in the quantity, form, and location of the FMRFamide-like immunoreactive cells. Thus, cells producing FMRFamide-like peptides were seen in all histologi-

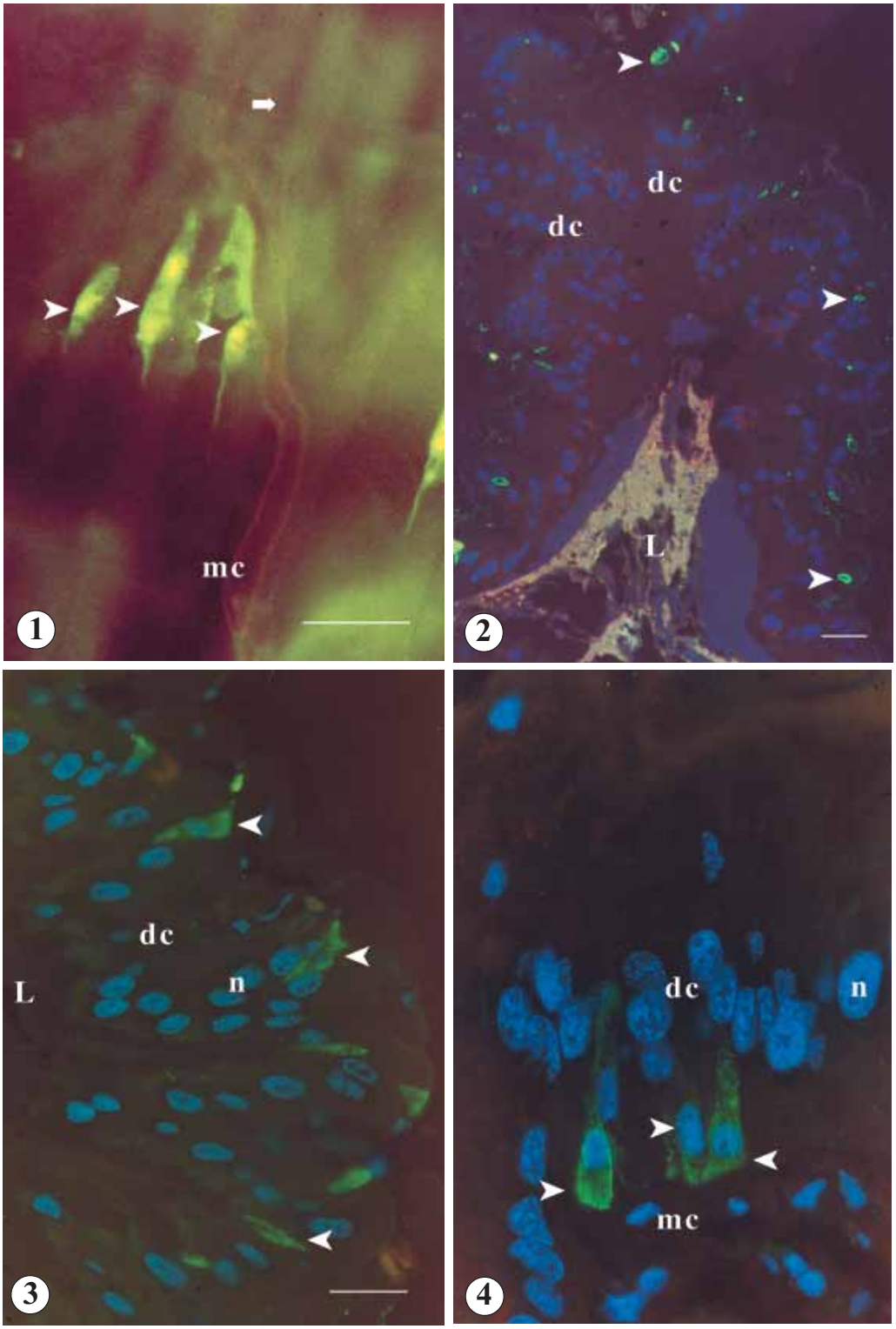

Figure 1. Whole mount showing FITC-labeled FMRFamide-like immunoreactive nerve cells in the posterior midgut of a forager worker. **Arrowheads** – immunoreactive nerve cells, **arrow** – trachea, **mc** – muscle cells. Bar = 30 µm.
Figure 2. FITC-labeled FMRFamide-like immunoreactive endocrine cells in the posterior midgut of a drone. **Arrowheads** – immunoreactive endocrine cells, **L** – lumen, **dc** – digestive cells. Section counterstained with DAPI. Bar = 30 µm.
Figure 3. FITC-labeled FMRFamide-like immunoreactive endocrine cells in the posterior midgut of a nurse worker. **Arrowheads** – immunoreactive endocrine cells, **L** – lumen, **dc** – digestive cells, **n** – nucleus. Section counterstained with DAPI. Bar = 30 µm.
Figure 4. FITC-labeled FMRFamide-like immunoreactive endocrine cells in the posterior midgut of a nurse worker. **Arrowheads** – immunoreactive cells, **n** – nucleus, **dc** – digestive cells, **mc** – muscle cells. Section counterstained with DAPI. Bar = 10 µm.

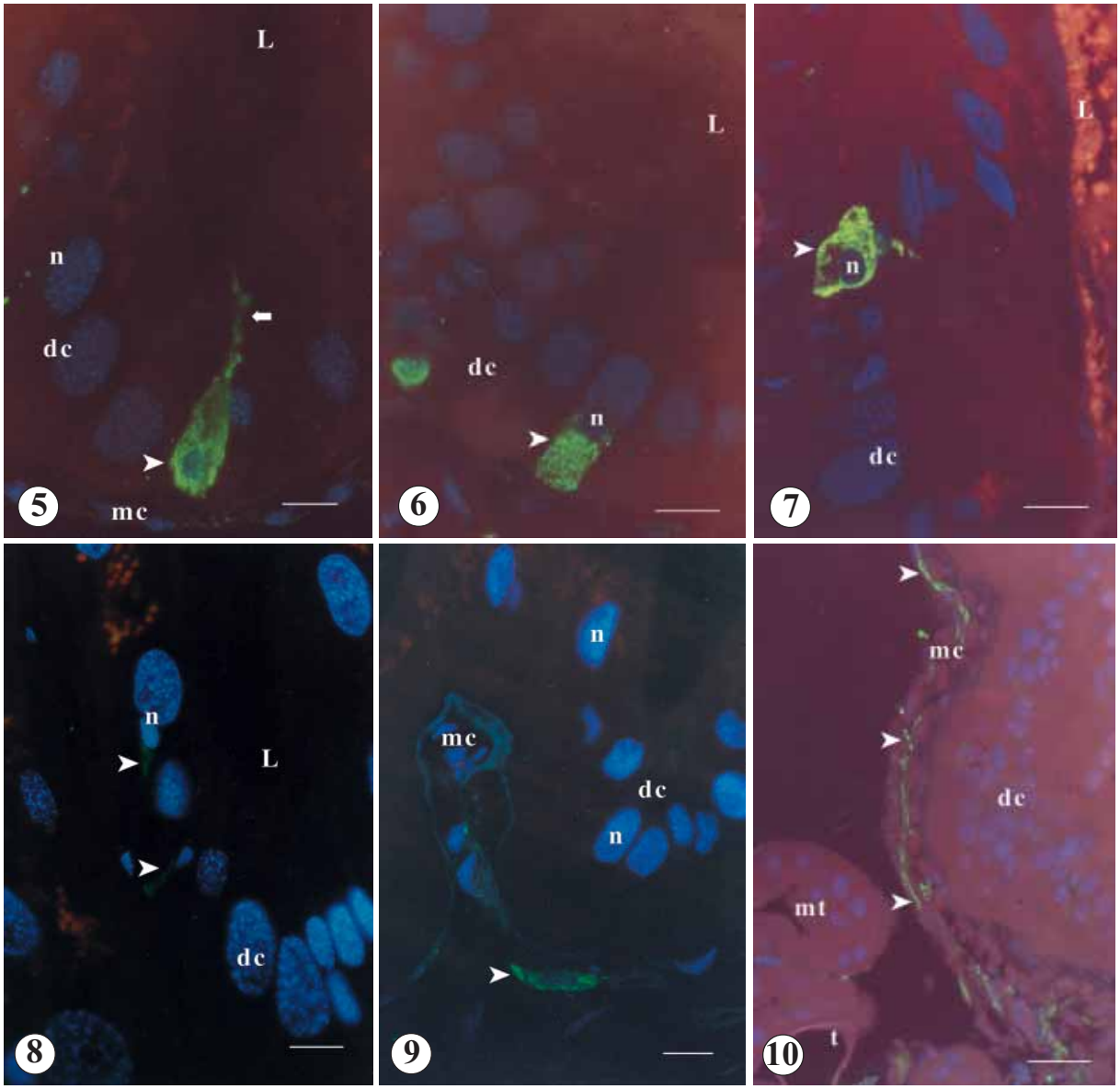

Figure 5. FITC-labeled FMRFamide-like immunoreactive endocrine cell in the posterior midgut of a queen. **Arrowhead** – "open" immunoreactive endocrine cell, **arrow** – cytoplasmic projection toward the lumen, **L** – lumen, **dc** – digestive cells, **mc** – muscle cells, **n** – nucleus. Section counterstained with DAPI. Bar = 10 µm.
Figure 6. FITC-labeled FMRFamide-like immunoreactive endocrine cell in the posterior midgut of a queen. **Arrowhead** – "closed" immunoreactive endocrine cell, **L** – lumen, **dc** – digestive cells, **n** – nucleus. Section counterstained with DAPI. Bar = 10 µm.
Figure 7. FITC-labeled FMRFamide-like immunoreactive endocrine cell in the posterior midgut of a forager worker. **Arrowhead** – large immunoreactive endocrine cell, **L** – lumen, **dc** – digestive cells, **n** – nucleus. Section counterstained with DAPI. Bar = 10 µm.
Figure 8. FITC-labeled FMRFamide-like immunoreactive endocrine cells in the posterior midgut of a forager worker. **Arrowheads** – two small, immunoreactive endocrine cells, **L** – lumen, **dc** – digestive cells, **n** – nucleus. Section counterstained with DAPI. Bar = 10 µm.
Figure 9. FITC-labeled FMRFamide-like immunoreactive nervous cell in the anterior midgut of a queen. **Arrowhead** – immunoreactive nerve cell, **dc** – digestive cells, **mc** – muscle cells, **n** – nucleus. Section counterstained with DAPI. Bar = 10 µm.
Figure 10. FITC-labeled FMRFamide-like immunoreactive nerve fibers in the posterior midgut of a queen. **Arrowheads** – immunoreactive nerve fibers, **dc** – digestive cells, **mc** – muscle cells, **mt** – Malpighian tubules, **t** – tracheole. Section counterstained with DAPI. Bar = 30 µm

cal sections of the posterior area of the midgut of queens, drones, and workers. The distribution of these cells throughout the midgut was variable. In some specimens, the cells were uniformly distributed in the posterior midgut, giving the impression that there was one FMRFamide-like immunoreactive cell per intestinal fold (Figs. 2 and 3). In other cases, the cells were observed in groups of three or more in a single fold

(Fig. 4), with no FMRFamide-like immunoreactive cells in the other folds. Nevertheless, the average number of positive cells in the terminal 1 mm of the midgut per caste was surprisingly similar [F (3; 68) = 0.574, p> 0.05] (Table 1).

Table 1. The number of FMRFamide-like immunoreactive cells (mean ± SD) in the posterior 1 mm end of the midgut in different castes of *Melipona quadrifasciata anthidioides* (Hymenoptera, Apidae, Meliponini).

Caste	Positive cells
Forager worker	32 ± 1
Nurse worker	31 ± 1
Queen	30 ± 1
Male	31 ± 1

The morphology of the FMRFamide-like immunoreactive endocrine cells was quite variable. All of the cells reached the basal membrane and the largest concentration of positive FMRFamide granules occurred in the infranuclear area. Most of the cells had a very thin prolongation that reached the intestinal lumen and also contained immunostained material; these cells were classified as "open" cells (Fig. 5). In other cells, the granules were clearly concentrated in the basal region and there was no prolongation in the direction of the gut lumen; these cells were classified as "closed" cells (Fig. 6). The FMRFamide-like immunoreactive cells were always smaller than the digestive cells, but their dimensions were extremely variable (compare Figs. 7 and 8). This variation in size occurred among castes and among specimens of the same caste.

A well-developed network of FMRFamide-like immunoreactive nerve fibers was observed in the posterior end of the midgut among the muscular cells, close to the confluence of the Malpighian tubules (Fig. 10). No FMRFamide-like immunoreactive midgut endocrine cells were seen in this region. A similar arrangement of nerve fibers was seen in the anterior region of the midgut.

DISCUSSION

There have been few studies of the midgut endocrine cells in the Hymenoptera. Somatostatin- and FMRFamide-producing cells were detected by immunocytochemistry in the midgut of *Apis* sp. [7] and *Bombus* sp. [61], and by immunofluorescence in the midgut of *M. quadrifasciata anthidioides* [41]. Structural and ultrastructural aspects of midgut endocrine cells have been described for *Apis mellifera* [32,46], and *Trigona spinipes* and *T. hypogea* [53].

Despite the high prevalence of FaRPs-like immunoreactive cells in the midgut of almost all insects studied so far, the function of these cells is not well understood [36]. FaRPs also occur in the nervous system of insects so that the action of these substances cannot always be attributed to a single source. For instance, Huang *et al.* [30] found that in *Helicoverpa zea* the concentration of FaRPs in the gut was greater than in the head. FaRPs detected in the hemolymph, could derive from neurohemal sources, as well as from the gut. The myotropic action of these peptides could explain their participation in reproduction, as observed in the cephalopod *Sepia officinalis* [29] and in *Rhodnius prolixus* [54]. In these species, FMRFamide stimulates muscular contraction of the oviduct and thus contributes to oviposition.

The influence of FaRPs in digestive processes can also be explained by their myotropic action on visceral muscle, which has been demonstrated *in vitro*, for many invertebrates [23,50] and mammals [14,47]. This myotropic action is fundamental for the control of the sphincter and peristalsis as a whole. FaRPs may also regulate other digestive processes, such as enzyme secretion and the control of water and electrolyte absorption [34,36,59,62].

The experiments done here did not allow us to quantify the content of FMRF amide-like immunoreactivity in each caste. In any case, such quantification can be complicated by the fact that antisera to FMRFamide frequently react with several FaRPs [49]. Although no qualitative differences were observed in the number, type and distribution of immunoreactive cells in each caste, a quantitative difference in the concentration of FaRPs, as observed in infested larvae of *Manduca sexta* [34], and in the concentration of locustatachykinin, in female and male *Locusta migratoria* [45], cannot be excluded. The similar numbers and forms of FMRFamide-like immunoreactive cells found in drones, queens, and workers suggest that a direct involvement of these cells in reproduction, such as occurs in *Rhodnius prolixus* [53] can be excluded even though nurse workers can lay eggs [6].

Almost no positive FMRFamide-like immunoreactive fibers were seen in areas where positive FMRFamide-like endocrine cells were concentrated.

Positive FMRFamide-like immunoreactive nerve fibers were concentrated near the cardiac and pyloric valves in the transition from the foregut to the midgut and from the midgut to the hindgut, respectively. It seems to be reasonable to suppose that in the midgut, where most of the digestion and absorption of nutrients occur in insects [58], the endocrine cells play a role in detecting of the luminal condition and in adapting the gut to such conditions, as in vertebrates [21,22]. Enzyme secretion, the absorption of nutrients, and the control of food flux are modulated by these conditions.

Open gut endocrine cells can "sense" the presence of food and nutrients, whereas closed gut endocrine cells can "sense" variations in gut volume [20]. The passage of food from the foregut to the midgut and from the midgut to the hindgut may have a more neuroendocrine than paracrine control. In bees, which need to regulate the passage of food through the proventriculus, the neural control of this flow would be useful. Cruz-Landim [13] found common axons and axons with ultrastructural characteristics of neuroendocrine fibers in the proventriculus of bees. The common axons would act in the control of food ingestion whereas the neurosecretory axons, which do not show typical synapses with muscle cells, would be responsible for controlling actions that last the bee's entire life, or for controlling the development of a specific task, such feeding the queen and/or larvae. The secretion of neurohormones directly into the hemolymph by exocytosis may guarantee a long-lasting action of these hormones [44].

The spatial differences observed here in the concentration of FMRFamide-like immunoreactive nerve fibers and endocrine cells in the foregut and hindgut and in most of the midgut have been found in many insect species [38,61] and may reflect variations in the embryological origins of these segments. The midgut has an endodermal origin, while the foregut and hindgut have an ectodermal origin [11]. In general, the nerve network which covers the gut wall in most insects derives from the proventricular ganglia or from a nerve plexus located at the foregut-midgut boundary with endings in the musculature of the foregut and midgut (gastric nerves). This gut nerve network also originates with proctodeal nerves that arise from the most caudal ganglion of the nerve cord and innervate the hindgut before proceeding towards the midgut-hindgut boundary where they mix with the nerve endings of the gastric nerves [11,51].

Many of the nerve fiber endings of the gastrointestinal tract are neurohemal areas, and contain many of the antigens observed in the central nervous system and endocrine cells [51]. The distribution of FMRF amide-like immunoreactive endocrine cells, neurons, and nerve fibers varies significantly among insects. FMRFamide-like immunoreactive nerve fibers of *Bombus* sp. and four species of Paraneoptera were not labeled, although in *Bombus* sp. about 20 nerve-cell bodies were observed into the wall of the anterior region of the midgut [61]. In *Ascalaphus* sp. (Neuroptera), some endocrine cells of the posterior area of the midgut are associated with nerve fibers [61].

The lack of labeling in FMRFamide-like immunoreactive nerve fibers and endocrine cells in certain regions of the midgut suggests important differences in the gut physiology of insects, but does not necessarily imply that these fibers and cells were absent. The presence of nerve fibers and endocrine cells could be detected through the use of other antibodies or techniques.

Only a few nerve-cell bodies and endocrine cells scattered throughout the midgut are observed in *Bombus* sp. [61], while in *M. quadrifasciata anthidioides* many FMRFamide-like immunoreactive nerve fibers were observed at both ends of the midgut, and FMRFamide-like immunoreactive endocrine cells were concentrated in the last one third of the posterior part of the midgut. This observation indicates that important variations in the distribution of nerve fibers and endocrine cells can occur in a same order and may reflect differences in the gut physiology of these insects.

In summary, there were no significant differences in the number, types, forms, and distribution of FMRFamide-like immunoreactive endocrine cells and immunoreactive nerve fibers in the midgut of different castes of *M. quadrifasciata anthidioides*. These results, together with other studies in the literature, suggest that FMRFamide and/or FaRPs are involved in the control of gut function in most insects, and may directly regulate the flow of food, enzyme secretion, and nutrient absorption by the gut.

ACKNOWLEDGMENTS

The authors thank Dr. L. M. Montuenga (Navarra University, Pamplona, Spain) for helpful comments on the use of the FMRFamide antiserum. This work was supported by the Brazilian research agencies CNPq, FAPEMIG and CAPES.

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Received: March 28, 2003
Accepted: July 10, 2003