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# Distributions of the endocrine cells in the gastrointestinal tract of nectarivorous and sanguivorous bats: A comparative immunocytochemical study

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ABSTRACT

The present study was conducted to clarify the influence of feeding habits on regional distribution and relative frequency of endocrine cells secreting cholecystokinin (CCK), gastrin (GAS), serotonin (5-HT) and enteroglucagon (GLUC) in the nectarivorous *Anoura geoffroyi* and *Glossophaga soricina* and the sanguivorous *Desmodus rotundus* bats of the Phyllostomidae family, by specific immunohistochemical methods. The regional distribution and frequency of the different types of endocrine cells varied according to their location in the GIT. 5-HT immunoreactive cells (IR), detected throughout the GIT of three bats, were the most predominant gastrointestinal endocrine cells. GAS-IR cells in *A. geoffroyi* were found at the base of the pyloric gland, while in *G. soricina* they could also be observed in the middle to basal portions of the gland. GLUC-IR cells were located in the fundic region of *A. geoffroyi*, *G. soricina* and *D. rotundus*. These endocrine cells were more abundant in the sanguivorous bat. In nectarivorous bats were compared to sanguivorous bat, which differ in dietary habits, difference in the distribution and relative frequency of gut endocrine cells would be predicted. The absence of some, and decrease in frequency of other, gastrointestinal endocrine cells may reflect, in part, its interspecies differences or dietary habits.


## 1. Introduction

The Chiroptera order is surpassed in number of species only by rodents. In Brazil, there are 138 known species to date, representing 74% of the total for South America (Koopman, 1993). Since bats show greatly divergent diets in a single order, the morphological adaptation of their digestive system to different modes of feeding has received a great deal of attention (Komori et al., 2000). The gastrointestinal tract (GIT) of these diverse mammals is a very attractive model not only for understanding the evolution of the digestive tract in general, but also for investigating the relationships between the distribution and frequency of gut endocrine cells and feeding habits (Yamada et al., 1987).

Today more than 30 gastrointestinal hormone genes and a multitude of GI hormones have been recognized, thus making the GIT the largest endocrine organs in the body (Ahlman and Nilsson, 2001). The gastrointestinal endocrine cells are dispersed along the epithelium, gastric and intestinal glands of the digestive tube and synthesize various types of biologically active polypeptides and amines (Sundler et al., 1980). The physiological functions mediated in the gut by these peptides include the control of motility, the secretion of fluid, electrolytes and digestive enzymes, cell proliferation and survival, vascular and immune functions, visceral pain and inflammation (Brown et al., 1973). Some GI dysfunctions are related to these hormones (Nyhlin et al., 1999). Some GI hormones have synergistic expression in gastric carcinoma and take part in the occurrence of gastric carcinoma (Milutinovic et al., 2003).

It is generally accepted that GI endocrine cells are remarkably different in regional distribution, relative frequency and cells types in the gastrointestinal tract (Huang and Wu, 2005). Some studies have elucidated the regional distribution and relative frequency of different endocrine cells in chiropteran's gastrointestinal tracts and 15 different kinds of these cells have been described using the immunohistochemical method. These studies have revealed some inter-species differences and suggest a certain correlation between endocrine cell distribution and feeding habits (Yamada et al., 1984, 1988; Ashihara et al., 1999; Komori et al., 2000).

The present study was conducted to clarify the influence of feeding habits on regional distribution and relative frequency of endocrine cells secreting cholecystokinin (CCK), gastrin (GAS), serotonin (5-HT) and enteroglucagon (GLUC) in the nectarivorous *Anoura geoffroyi* and *Glossophaga sorina* and the sanguivorous

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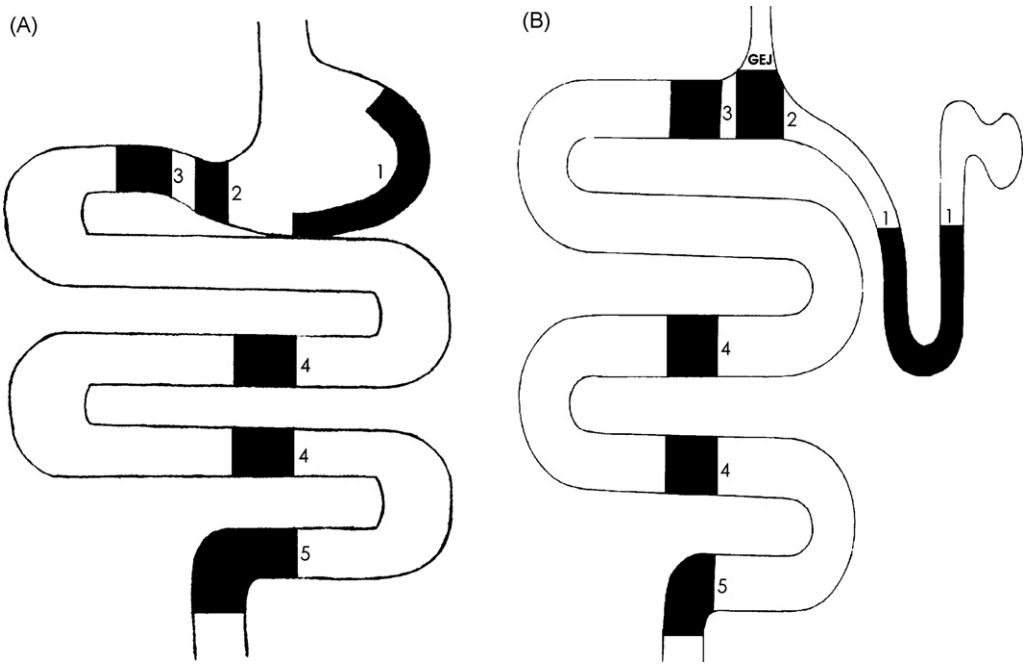


**Fig. 1.** (A and B) Representation of the digestive tract illustrating regions sampled from (A) nectarivorous and (B) sanguivorous. (1) Stomach, fundic region. (2) Stomach, pyloric region. (3) Intestine I, duodenum. (4) Intestine II, jejunum/ileum. (5) Intestine III, large intestine and rectum. (GEJ) Gastroesophageal junction (modified from Komori et al., 2000).

*Desmodus rotundus* bats of the Phyllostomidae family, by specific immunohistochemical methods, contributing to the knowledge of the cellular composition of the bat gastrointestinal tract.

## 2. Materials and methods

### 2.1. Animals and tissue preparations

Nine animals were used; three of them were *A. geoffroyi* (two males and one female), three *G. soricina* (three males) and three *D. rotundus* (two females and one male) collected according to Brazilian law. The specimens were collected during the night with mist nets and hand nets, in Casa de Pedra cave in the state of Sergipe, Brazil. The bats were sacrificed with sodium thiopentone at a dose of 100 mg/kg and the two regions of the stomach and three regions of the intestine were removed (Fig. 1A and B) and fixed with Bouin's fluid for 6 h. The tissues were dehydrated through a graded series of ethanol solutions and embedded in Histosec (Merk, Darmstadt, Germany) using routine protocols. 5-μm thick sections were cut by microtome and mounted on glass slides pre-coated with 0.1% poly-L-lysine (Sigma Chemical Co., Saint Quentin Fallavier, France).

### 2.2. Immunohistochemistry

The primary antisera were used for both the specificity controls and immunolocalization of cells immunoreactive to regulatory peptides and biogenic amine. They were: rabbit polyclonal anti-5-HT (S 5545—Sigma–Aldrich, Inc.), rabbit polyclonal anti-GAS (G 0785—Sigma–Aldrich, Inc.), rabbit polyclonal anti-CCK (C

**Table 1**
Distribution and relative frequency of endocrine cells of (A) the nectarivorous bats *A. geoffroyi** and *G. soricina* and (B) sanguivorous bat *D. rotundus* (cells/0.25mm$^2$, mean ± S.D.).

| Portion of alimentary tract | Serotonin | Gastrin | CCK | Enteroglucagon |
|---|---|---|---|---|
| (A) | | | | |
| Stomach | 11.2 ± 2.6*/24.3 ± 4.9 | f/0 | 0/0 | 2.2 ± 1.0/1.6 ± 1.0 |
| Fundic | | | | |
| Stomach | 29.7 ± 12.9/36.3 ± 8.1 | 48.2 ± 15.7/47.7 ± 4.3 | 0/0 | 0/0 |
| Pylorus | | | | |
| Intestine I | 15.4 ± 3.7/27.1 ± 14.3 | 0/0 | 9.6 ± 1.5/12.3 ± 2.0 | 2.8 ± 0.7/3.4 ± 1.0 |
| Intestine II | 10.3 ± 2.0/28.6 ± 9.0 | 0/0 | 11.9 ± 4.8/11.4 ± 1.4 | 2.0 ± 0.6/2.0 ± 0.5 |
| Intestine III | 16.9 ± 1.1/19.5 ± 6.5 | 0/0 | 11.5 ± 5.1/12.7 ± 6.7 | 2.4 ± 0.5/3.6 ± 1.9 |
| (B) | | | | |
| Stomach | 58.5 ± 7.2 | 0 | 0 | 4.0 ± 1.2 |
| Fundic | | | | |
| Stomach | 45 ± 5.0 | 26.7 ± 6.0 | 0 | 0 |
| Pylorus | | | | |
| Intestine I | 18.8 ± 6.4 | 0 | 10 ± 2.2 | 3.0 ± 1.7 |
| Intestine II | 10.9 ± 3.0 | 0 | 14.7 ± 5.2 | 1.8 ± 0.8 |
| Intestine III | 13.5 ± 3.0 | 0 | 7.1 ± 2.8 | 2.2 ± 0.8 |

f: few—not detected in every animal.

2581—Sigma–Aldrich, Inc.) and mouse monoclonal anti-glucagon (GLUC) (G 2654—Sigma–Aldrich, Inc.). We refer to the endocrine cells immunoreactive to glucagon antiserum with code G 2654 as enteroglucagon-immunoreactive cells, since the antiserum showed a cross-reaction with pancreatic glucagon and enteroglucagon.

The sections were dewaxed and rehydrated by routine protocols. They were incubated with methanol containing 0–3% $H_2O_2$ for 15 min to block any endogenous peroxidase. The sections were then incubated with a 1:100 dilution of bovine serum albumin (B4287; Sigma) in phosphate buffered saline (PBS) solution for 30 min. Subsequently, they were labeled immunohistochemically using a three layered avidin–biotin–peroxidase complex (ABC) method (Hsu et al., 1981) to identify specific endocrine cells. The sections were first incubated overnight at 4 °C with the primary antisera against individual gastrointestinal hormones, at the following dilutions: 1:8000 for 5-HT; 1:1000 for GAS; 1:8000 for CCK; 1:2000 for glucagon (GLUC). The sections were then incubated with biotinylated "Universal" secondary antibody diluted to 1:200 (PK 7200, Vector Laboratories, Inc., UK) for 30 min, then with ABC, diluted at 1:200, for 30 min (both from PK 6200, Vector Laboratories, Inc.). Subsequently, the peroxidase label was revealed by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Dakocytomation 003222, CA, USA) prepared according to the kit instructions. All steps were performed at room temperature unless otherwise spec-

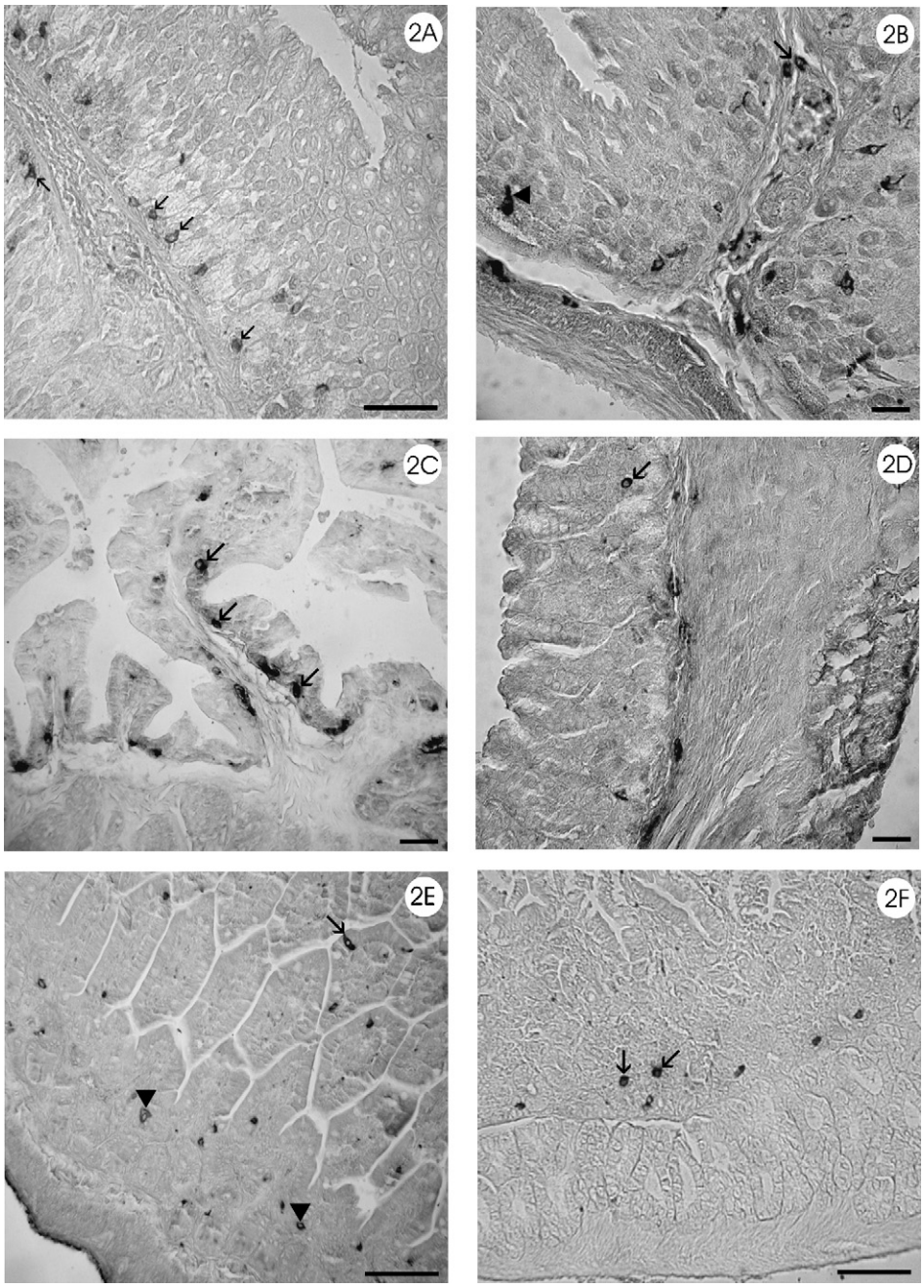


**Fig. 2.** (A–F) Photomicrographs of cells immunoreactive to Serotonin/5-HT. (A) Stomach of *G. soricina* with markings in the middle and basal portion of the fundic gland (arrows). (B) Stomach of A. *geofroyi* with piriform cells with an apical cytoplasmic process (arrowhead) and markings in the submucosa among the components of the connective tissue (arrows). (C) Stomach of *Desmodus rotundus*, fundic region. Note the markings in the inter-epithelial part of the basal portion of the epithelial lining (arrows). (D) Pyloric region of *A. geoffroyi* with oval cells in the basal half of the glands (arrows). (E) Intestine II of *D. rotundus* with markings on the surface epithelium (arrow) and intestinal glands (arrowhead). (F) Intestine I of *A. geoffroyi*. Note the markings on the intestinal gland (arrows). Scale bar = 50 μm.

ified. All dilutions and thorough washes between stages were performed in PBS unless otherwise specified. The slides were finally rinsed several times with deionized water, dehydrated through a series of ethanol solutions and methylcyclohexanes, and mounted using Entelan (Merck).

### 2.3. Controls

In the present study, the immunocytochemical location of regulatory peptides and biogenic amine 5-HT inside in the endocrine cells was investigated by use of polyclonal and monoclonal antiserum. The control method was demonstrated by the usual specificity tests, which included: (1) omission of the primary antiserum; (2) replacement of the primary antiserum with non-immune serum; (3) dilution profiling of the primary antiserum using doubling dilutions on serial sections; (4) assessment of the influence of the salt content (up to 0.5 M) of the buffer; (5) complement-deprived antisera (Heyderman, 1979; Van Noorden, 1986; Burry, 2000).

### 2.4. Observation, photomicrography and cell count

Three samples from each of the nine bats were observed using an Olympus Dx-41 photomicroscope and representative images were captured. The relative frequency of immunoreactive (IR) cells in each region was calculated as the number of IR cells per unit area (0.25 $mm^2$) of tissues using a computerized image analyzer (Image Pro-Plus software). The frequency of immunoreactive cells is expressed as mean + S.D. (standard deviation) per unit area.

## 3. Results

5-HT-, GAS- and GLUC-IR were identified in the stomach, and 5-HT-, CCK-, GLUC-IR were identified in the intestine of *A. geoffroyi*, *G. soricina* and *D. rotundus*. The regional distribution and frequency of the different types of endocrine cells varied according to their location in the GIT. These differences are shown in Table 1. No positive labeling was seen in any of the negative control sections.

### 3.1. 5-HT-immunoreactive cells

5-HT-IR cells, detected throughout the GIT of three bats. In the stomach of the nectarivorous bats *A. geoffroyi* and *G. soricina*, they were located in the middle and basal portion of the fundic gland (Fig. 2A). These endocrine cells were piriform in shape and frequently had an apical cytoplasmic process directed towards the glandular lumen, and was therefore classified as open types (Fig. 2B). These 5-HT immunoreactive cells were also detected in the submucosa among the components of the connective tissue (Fig. 2B). In the sanguivorous bat *D. rotundus*, the 5-HT-IR cells were situated in the surface epithelium of the fundic region with highest frequency ($58.5 \pm 7.2$ cells/0.25 $mm^2$) (Fig. 2C). In the pyloric region of the nectarivorous bats they were found primarily in the basal half of the glands and were frequently oval in shape (Fig. 2D). In

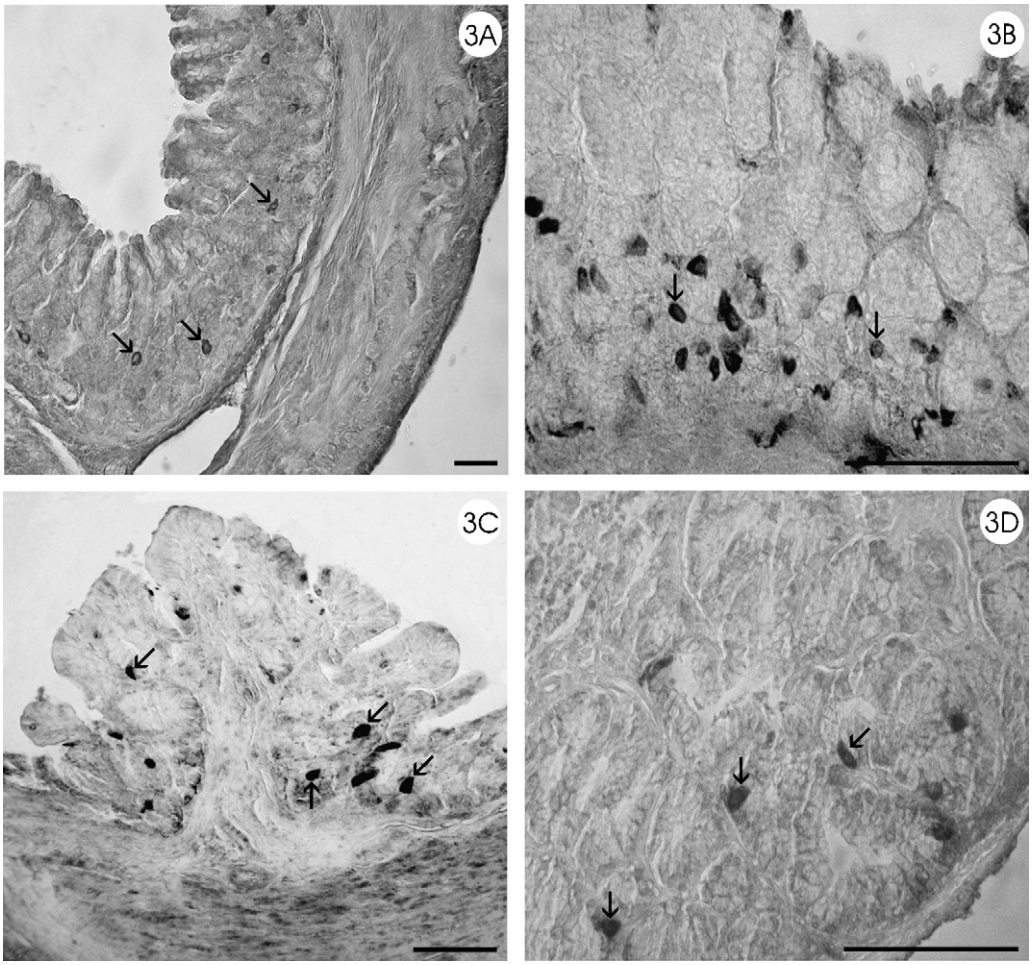


**Fig. 3.** (A–D) Photomicrographs of cells immunoreactive to gastrin in the stomach and fundic region. (A) *A. geoffroyi* with markings in the middle to basal portions of the fundic gland (arrows). (B) *G. soricina* with oval and closed type IR-cells (arrows). (C and D) *D. rotundus* with markings in the inter-epithelial part of the basal portion of the epithelial lining (arrows). Scale bar = 50 μm.

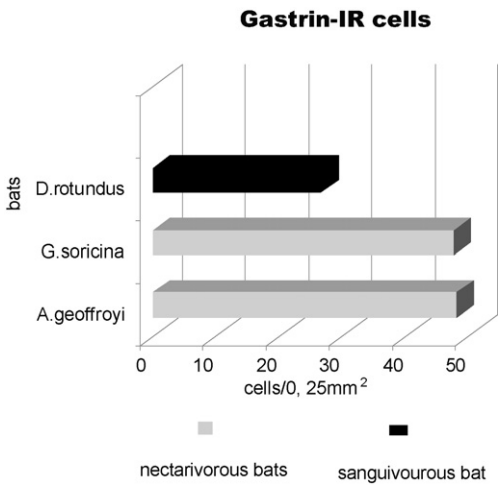


**Fig. 4.** Relative frequency of the gastrin immunoreactive cells in sanguivorous (*D. rotundus*) and nectarivorous (*G. soricina* and *A. geoffroyi*) bats.

this region of the sanguivorous bat this IR cell was observed both in the surface epithelium and pyloric gland. Their relative frequency varied along the intestine in the species studied. They were mostly found in the surface epithelium and intestinal glands (Fig. 2E). These endocrine cells were piriform in shape. In the Brunner's glands the 5-HT-IR cells were not detected (Fig. 2F).

### 3.2. Gastrin-immunoreactive cells

GAS-IR cells in *A. geoffroyi* were found at the base of the pyloric gland, while in *G. soricina* they could also be observed in the middle to basal portions of the gland (Fig. 3A). They were frequently oval in shape and did not present a cytoplasmic process, so were classified as of the closed type (Fig. 3B). In the sanguivorous bat the relative frequency was lower than in the two nectarivorous bats, showed in Fig. 4, and they were found only in the pyloric gland with irregular shape (Fig. 3C and D).

### 3.3. CCK-immunoreactive cells

CCK-IR cells were detected in the gut of the nectarivorous bats and the sanguivorous species. They were more abundant in the intestine II of *D. rotundus* ($14.7 \pm 5.2$ cells/0.25 mm$^2$). These endocrine cells were situated in the surface epithelium and also in the glands of intestine I–III (Fig. 5A and B). They were mainly piriform with the presence of a cytoplasmic process (Fig. 5C). These endocrine cells were also detected in the Brunner's glands of the *A. geoffroyi*.

### 3.4. Glucagon-immunoreactive cells

These IR-cells were located in the fundic region of *A. geoffroyi*, *G. soricina* and *D. rotundus*. They were more abundant in the sanguivorous bat ($4.0 \pm 1.2$ cells/0.25 mm$^2$). In the two nectarivorous species

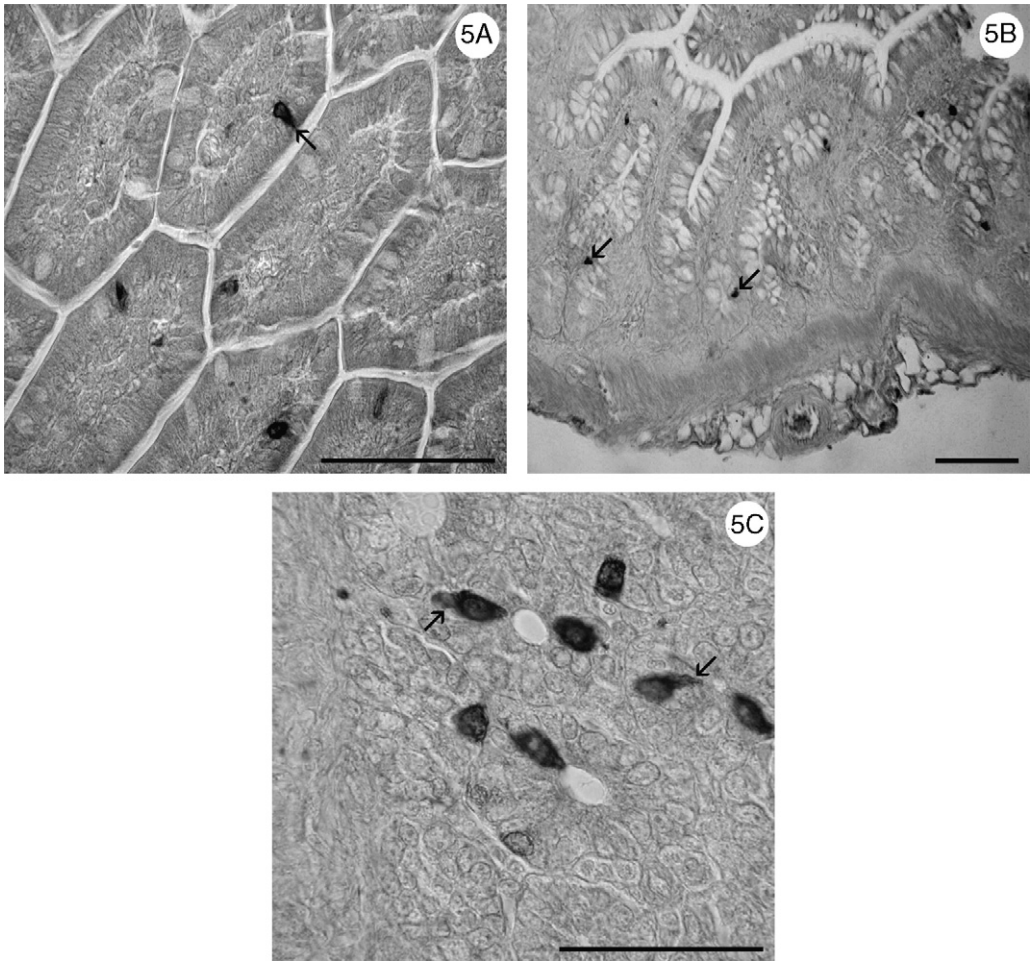


**Fig. 5.** (A–C) Photomicrographs of cells immunoreactive to CCK. (A) Intestine II of *D. rotundus* with inter-epithelial markings (arrows). (B) Intestine III of *G. soricina*. Note the markings on the surface epithelium and also in the intestinal glands (arrows). (C) Intestine II of *A. geoffroyi* with piriform cells with the presence of a cytoplasmic process (arrows). Scale bar = 50 μm.

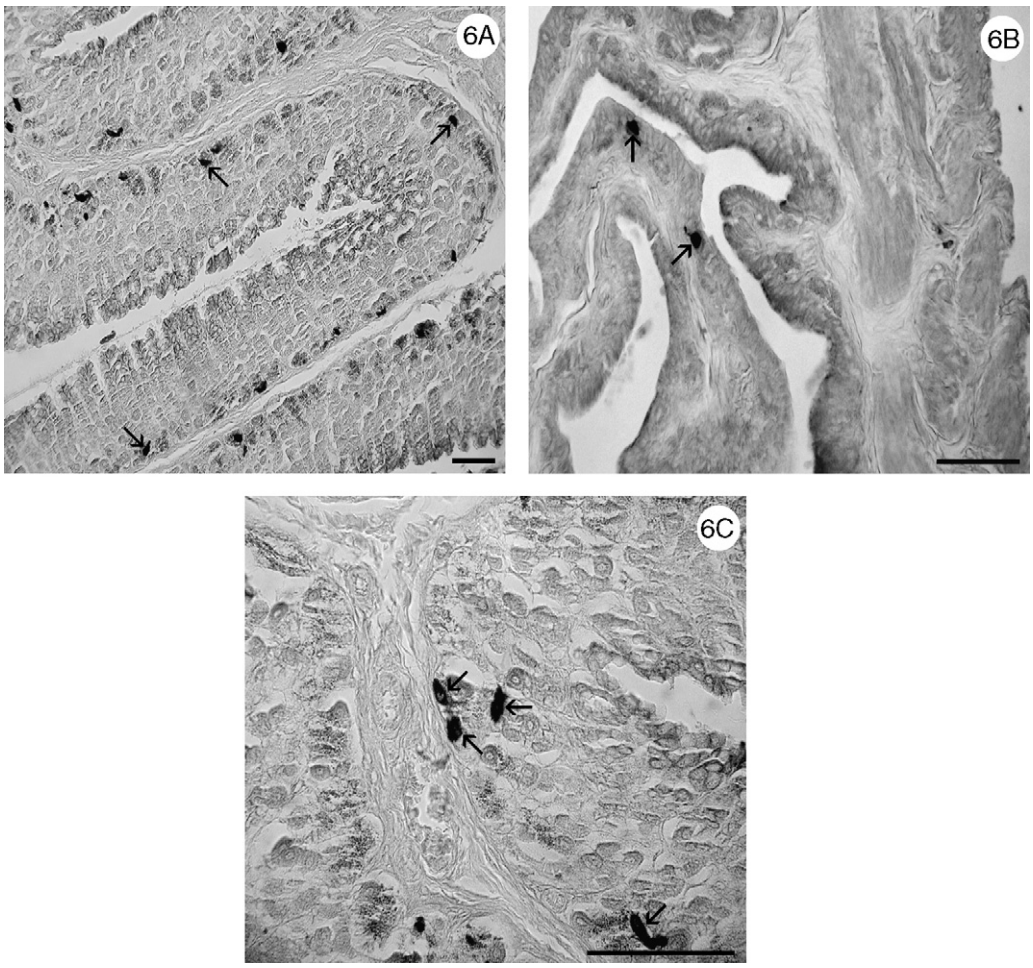


**Fig. 6.** (A–C) Photomicrographs of cells immunoreactive to glucagon. (A) Stomach of *G. soricina* with distribution at the base of the fundic gland (arrows). (B) Stomach of *D. rotundus* with markings on the surface epithelium (arrows). (C) Fundic glands of *A. geoffroyi* with markings on the basal portion of the gland and irregular shaped cells (arrows). Scale bar = 50 μm.

they were distributed in the base of the fundic gland (Fig. 6A), but only in the surface epithelium in *D. rotundus* (Fig. 6B). Their predominant shape in all three species was irregular (Fig. 6C). As GLUC-IR cells were observed along the entire intestine. Their relative frequency was greatest in intestine I of *G. soricina* ($3.4 \pm 1.0$ cells/0.25 $mm^2$) and *D. rotundus* ($3.0 \pm 1.7$ cells/0.25 $mm^2$), and their most frequent shape was oval, without a cytoplasmic process, which classifies them as of the closed type.

## 4. Discussion

Preliminary studies in our laboratory have revealed the distribution and relative frequency of gastrointestinal endocrine cells in insectivorous (*Lonchorhina aurita* and *Molossus molossus*) (Santos et al., 2008a) and frugivorous species (*Artibeus cinerius* and *Sturnira lilium*) (Santos et al., 2008b) using immunohistochemical methods. Thus, it is tempting to compare those previous results in insectivorous vespertilinid *Pipistrellus abramus* and *Plecotus auritus sacrimontis* (Yamada et al., 1988), frugi-nectarivorous and nectarivorous phyllostomid (*Anoura caudifer* and *Carollia perspicillata*) (Ashihara et al., 1999) and piscivorous (*Noctilio leporinus*) (Komori et al., 2000) bats.

The noteworthy differences 5-HT is a widespread aminergic constituent of the matrix of secretory granules in the diffuse gastro-enteropancreatic (GEP) neuroendocrine system of mammals and other vertebrates, where it is co-stored and co-secreted with specific corresponding hormones (Maake et al., 2001), neuropeptides (D'Este et al., 1994, 1995; Trandaburu et al., 1999) and granin proteins (Lee and Ku, 2004). According to the current concept, the GEP organs represent the second major source of 5-HT (behind only the central nervous system) and are also important targets for this monoamine (for references see Frazer and Hensler, 1999). Serotonin-secreting cells were the most frequent endocrine cells in the species studied here. They were more abundant in the stomach than in the gut. These cells were also observed in the stomach and intestine of the nectarivorous marsupial *Tarsipes rostratus* (Yamada et al., 1989). In the sanguivorous bat *Desmodus rotundus,* these IR-cells were more frequently found in the fundic region, unlike the results obtained insectivorous (Santos et al., 2008a), frugivorous (Santos et al., 2008b) and piscivorous bats (Komori et al., 2000), in which these cells were mainly found in the pyloric region. These IR-cells were abundant along the entire length of the intestine in all species of bats studied here, having been observed in the surface epithelium and the intestinal glands. These cells were not detected in the Brunner's glands in *G. soricina*, *A. geoffroyi*, *D. rotundus*. The same is true for the piscivorous *N. leporinus* (Komori et al., 2000) and insetivorous *P. auritus sacrimontis* (Yamada et al., 1988), but they have been identified in the nectarivorous *Anoura caudifer*, *Carollia perspicillata* (Ashihara et al., 1999), insetivorous *P. abramus, M. molossus* (Yamada et al., 1988; Santos et al., 2008a) and frugivorous *S. lilium* (Santos et al., 2008b).

The gastric hormone gastrin stimulates gastric acid secretion and epithelial cell proliferation (Dockray, 2004). Recent findings indicate that gastrin, acting via CCK-2 receptors, regulates the

expression of a number of genes in gastric mucosa that potentially influence the organization of the mucosa, including a trefoil factor, matrix metalloproteinases and inhibitors of extracellular proteolysis (Wroblewski et al., 2002). Recent work indicates that non-amidated gastrins stimulate the proliferation of a variety of tumor cells through mechanisms independent of the CCK-2 receptor (Seva et al., 1994; Singh et al., 2003). These IR-cells were found in moderate frequency in the nectarivorous bats *G. soricina* and *A. geoffroyi* and in less frequency in the sanguivorous *D. rotundus*. Previous investigations have revealed that fruit bats have abundant, highly active acid-producing parietal cells (Forman, 1972). With this in mind, it is interesting that the two frugivorous bats, *A. cinerius* and *S. lilium* have a significantly dense population of GAS-IR cells (126.9 and 75.8 cells/0.25 $mm^2$) (Santos et al., 2008b). Mennone et al. (1986) observed that the nectarivorous bat *G. soricina* has moderately active parietal cells, which can justify the moderate frequency of the GAS-IR cells found in the nectarivorous bats in this study. In the sanguivorous bat *D. rotundus* there were fewer GAS-IR cells observed than in the other species studied. This difference might not only be related to the low activity of the parietal cells. It might reveal differences in the ways that sanguivorous bats obtain nutrients and subdivide nutrient resources in the tropics (Mennone et al., 1986).

CCK is a peptide hormone discovered in the small intestine. CCK, secretin and gastrin constitute the classic gut hormone triad. In addition to gallbladder contraction, CCK also regulates pancreatic enzyme secretion and growth, intestinal motility, satiety signaling and the inhibition of gastric acid secretion. CCK is, however, also a transmitter in central and intestinal neurons (Rehfeld, 2004). The most remarkable difference concerns CCK-immunoreactive cells. These cells were observed scattered along the entire length of the intestine of the nectarivorous and sanguivorous bats studied here. A similar distribution has been reported in frugivorous (Santos et al., 2008b), insectivorous bats (Yamada et al., 1988; Santos et al., 2008a) and in the Virginia opossum and nectarivorous marsupial honey possum (Yamada et al., 1989; Krause et al., 1985). However, CCK-immunoreactive cells have not been detected in piscivorous (Komori et al., 2000), nectarivorous and frugi-nectarivorous bats (Ashihara et al., 1999). Komori et al. (2000) suggested that these results reflect differences in feeding habits or may reflect a real absence. However, *Anoura caudifer* and *Anoura geoffroyi* belong to the same genera and have similar feeding resources. Perhaps our findings do not reflect a real difference regarding the frequency of these IR-cells in species that are so close phylogenetically, but rather differences in the methodologies employed or the antisera used.

Glucagon produced in the Langerhans islets of vertebrates is also found in the digestive tract of lower vertebrates. The cells containing this peptide are gradually affected by the pancreas during embryonic development. Probably no relationship exists, however, between pancreatic and gastric immunoreactive cells. Earlier reports showed that this peptide is secreted paracrinally and has a proliferative function (Noaillac-Depeyre, 1985; Elbal and Agulleiro, 1986). In the present study, the relative frequency of enteroglucagon-immunoreactive cells was moderate, similar to the findings reported in nectarivorous, frugi-nectarivorous (Ashihara et al., 1999) and insectivorous bats (Yamada et al., 1988). The distribution of enteroglucagon-immunoreactive cells was similar to that of frugi-nectarivorous (Ashihara et al., 1999), frugivorous (Santos et al., 2008b) and piscivorous bats (Komori et al., 2000), where these IR-cells have been observed only in the fundic region of the stomach and along the intestine.

In nectarivorous *G. soricina* and *A. geoffroyi* bats were compared to sanguivorous *D. rotundus* bats, which differ in dietary habits, difference in the distribution and relative frequency of gut endocrine cells would be predicted. The absence of some, and decrease in frequency of other, gastrointestinal endocrine cells may reflect, in part, its interspecies differences or dietary habits (Ashihara et al., 1999).

Therefore, the present study reinforces the hypothesis done by Yamada et al. (1989), that they suggest the correlation between the distributions of endocrine cells regulating gastrointestinal function and feeding habits and these differences might help us understand the evolution of the digestive tract in relationship to the partitioning by nutrient resources and evolutional niches.

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