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A comparative immunohistochemical study of endocrine cells in the digestive tract of two frugivorous bats: *Artibeus cinerius* and *Sturnira lilium*

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Summary

The purpose of the present study was to examine the serotonin (5-hydroxytryptamine, 5-HT), gastrin (GAS), cholecystokinin (CCK) and glucagon (GLUC) endocrine cells in the gastrointestinal tract of frugivorous Phillostomidae bats, *Sturnira lilium* and *Artibeus cinerius*, to clarify the correlation between distribution of cell types and their relative frequency, with feeding habits. Five portions of the gastrointestinal tract – fundus, pilorus, and three parts of the intestine, I, II and III – were examined. Most of the immunoreactive cells in the stomach and intestine were of triangular, oval or piriform shape. Serotonin-immunoreactive cells were most commonly found in the *S. lilium* intestine I (66.6 ± 9.9) and the *A. cinerius* intestine III (35 ± 18). Gastrin-immunoreactive cells were the most abundant cell type in the pyloric glands of both species. They were more numerous in *A. cinerius* (126.9 ± 27.4) than in *S. lilium* (75.8 ± 1.8). CCK-immunoreactive cells were found in the alimentary tract epithelia at moderate frequencies in both species. GLUC-immunoreactive cells were detected at very low or low frequencies. This study suggests that there is a correlation between endocrine cell distribution and frequency, and the feeding habits of the bats.

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Introduction

Gastrointestinal (GI) endocrine cells are dispersed along the epithelia and glands of the gastrointestinal tube (GIT). They produce various types of hormones and play important roles in the physiological functions of the GIT (Bell, 1978).

Secretin, gastrin (GAS) and cholecystokinin (CCK) were the first gut hormones to be described. Currently, more than 30 genes coding for GI hormones are recognized, along with a multitude of bioactive peptides, which make the gut the largest endocrine organ in the body (Ahlman and Nilsson, 2001). In recent years, the field of GI hormones has expanded rapidly and has become an important domain in gastroenterology (Huang and Wu, 2005). Some GI dysfunctions are related to GI hormones (Milutinovic et al., 2003). The investigation of endocrine cells is considered an important part of GI hormone studies and can provide baseline data for basic research into gastroenterology (Huang and Wu, 2005).

The Chiroptera order is surpassed in number of species only by rodents. It comprises 17 families, 177 genera and nearly 930 species (Wilson and Reeder, 1993). The Chiroptera GIT is a very attractive GI model, not only for understanding the digestive tube's evolution in general, but also for investigation of the relationship between the distribution and frequency of gut endocrine cells and animal feeding habits. This is possible because bats have very varied diets. Carnivorous, insectivorous, frugivorous, nectarivorous and sanguivorous species can be compared (Yamada et al., 1988).

More than 15 different endocrine cell types have been described in bats' digestive tracts (Yamada et al., 1984, 1988; Ashihara et al., 1999; Komori et al., 2000; Santos et al., in press). These studies have revealed some inter-species differences in the frequency, cells types and regional distributions of different endocrine cells in the GIT of bats. They highlight the need to pay much more attention to the classificatory position of specific bat species and to compare the possible effects of feeding habits on the distribution and frequency of gut endocrine cells.

The purpose of the present study was to examine, for the first time, the serotonin, GAS, CCK and glucagon (GLUC) endocrine cells in the GIT of frugivorous Phillostomidae bats, *Sturnira lilium* and *Artibeus cinerius*, using immunohistochemistry, to elucidate the correlation between the distribution of these cells, their relative frequency and the feeding habits of the bats.

Materials and methods

Six animals were studied; three *A. cinerius* (two males and one female) and three *S. lilium* (two males and one female). The animals were collected according to Brazilian laws (license 042/2005-Process number 02001, 007915/01/72 /IBAMA). The bats were caught at night with mist nets and hand nets in the Tinguá Reserve in the municipality of Nova Iguaçu, Rio de Janeiro State, Brazil. The bats were sacrificed with sodium thiopentone at a dose of 100 mg/kg. The GIT were removed and fixed with Bouin's fluid for 6 h. After fixation, the tubes were separated into the five regions as shown in Figure 1. The tissues were dehydrated through a graded series of ethanol solutions and embedded in Paraplast using routine protocols. Five-micrometer thick sections were cut by microtome and mounted on glass slides precoated with 0.1% poly-L-lysine (Sigma Chemical Co., Saint Quentin Fallavier, France).

Primary antisera

The primary antisera were used for both the specificity controls and immunolocalization of cells

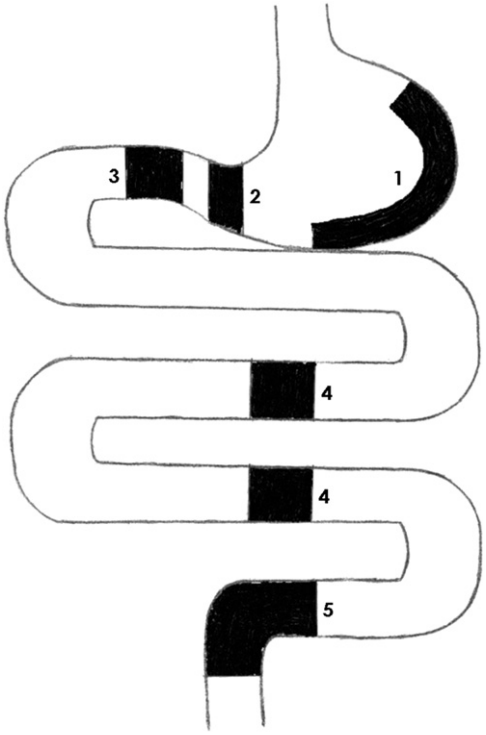

Figure 1. Schematic drawing of the digestive tract illustrating the regions sampled from *A. cinereus* and *S. lilium* (modified from Komori et al., 2000). Stomach, fundic region, 2. Stomach, pyloric region, 3. Intestine I – duodenum, 4. Intestine II, jejunum/ileum, 5. Intestine III – large intestine and rectum.

immunoreactive to regulatory peptides and biogenic amine. They were: rabbit polyclonal anti-serotonin (5-hydroxytryptamine, 5-HT) (S 5545-Sigma-Aldrich, Inc.), rabbit polyclonal anti-GAS (G 0785-Sigma-Aldrich, Inc.), rabbit polyclonal anti-colecystokinin (CCK) (C 2581-Sigma-Aldrich, Inc.) and mouse monoclonal anti-GLUC (G 2654-Sigma-Aldrich, Inc.). We refer to the endocrine cells immunoreactive to GLUC antiserum with code G 2654 as enteroglucagon-immunoractive cells since the antiserum showed a cross-reaction with pancreatic GLUC and enteroglucagon.

Single antigen immunohistochemistry

The sections were dewaxed and rehydrated by routine protocols. They were incubated with methanol containing 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), 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 GI hormones, at the following dilutions: 1:8,000 for serotonin (5-HT); 1:1,000 for GAS; 1:8,000 for CCK and 1:2,000 for GLUC. Sections were then incubated with biotinylated anti-horse serum, diluted 1:200, for 30 min, then with avidin–biotin complex (ABC), diluted 1:200, for 30 min (both from PK 6200, Vector Lab. Inc.). Subsequently, the peroxidase label was revealed by reaction with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) (Dakocytomation 003222) prepared according to kit instructions. All steps were performed at room temperature unless otherwise specified. 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).

Controls

In the present study, the immunohistochemical localization of regulatory peptides and biogenic amine (serotonin/5-HT) in the endocrine cells was investigated by use of polyclonal antiserum. The method control was demonstrated by the usual specificity tests, that 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, and (5) complement-deprived antisera (Heyderman, 1979; Van Noorden, 1986; Burry, 2000).

Observation, photomicrography and cell count

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

Results

Serotonin/5-HT-, GAS- and GLUC-immunoreactive cells were identified in the stomach, and serotonin/5-HT-, CCK- GLUC-immunoreactive cells were identified in the intestine of *A. cinerius* and *S. lilium*. 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.

Serotonin/5-HT-immunoreactivity

Serotonin/5-HT-immunoreactive cells, detected throughout the whole GIT of the two bat species, were the most commonly seen GI endocrine cells. They were found primarily in the basal half of the fundic and pyloric glands of *A. cinerius*, illustrated in Figure 2A. These serotonin/5-HT-immunoreactive cells were triangular or oval in shape, and were also detected in the submucosa among the components of the connective tissue, seen in Figure 2B. In *S. lilium*, the serotonin/5-HT-immunoreactive cells in the stomach were distributed mainly in the medial and basal portion of the fundic glands, seen in Figure 2C, and were frequently oval and triangular in shape, as illustrated in Figure 2D. In the pylorus region, they were concentrated mainly in the apical to medial region of the gland, seen in Figure 2E. Their relative frequency varied along the

Table 1. Distribution and relative frequency of endocrine cells of *A. cinerius** and *S. lilium* (cells/mm^2, mean ± SD)

Region	Serotonin	Gastrin	CCK	Enteroglucagon
Stomach-fundus	20.5 ± 1.7*/12.2 ± 3.4	0/0	0/0	0.91 ± 0.3/−
Stomach-pylorus	16.6 ± 5/14.6 ± 2.4	126.9 ± 27/75.8 ± 5.7	0/0	0/0
Intestine I	19.6 ± 7.5/66.6 ± 9.9	0/0	7.2 ± 1.8/24.1 ± 6.1	0/5.0 ± 2
Intestine II	18.6 ± 6.3/31.9 ± 9	0/0	8.2 ± 1.9/22.8 ± 8.5	1.7 ± 1/3.7 ± 1
Intestine III	35 ± 15/23.9 ± 0.6	0/0	14.2 ± 5.8/22.4 ± 5.5	3.7 ± 1/ 2.0 ± 0.5

intestine. These cells were at their highest density in the intestine III of *A. cinerius* (35 ± 18 cells/0.25 mm^2) and intestine I of *S. lilium* (66.6 ± 9.9/0.25 mm^2). They were mostly found in the intestinal epithelium and intestinal glands. These endocrine cells were piriform in shape and frequently had apical cytoplasmic process directed towards the glandular lumen, and were therefore classified as an open type (Figure 2F). In *S. lilium* sparse serotonin/5-HT-immunoreactive cells were detected in the Brunner's glands, located in the submucosa of the pyloro-duodenal junction.

Gastrin immunoreactivity

GAS-immunoreactive cells were most abundant in the pyloric glands in both species of bats. In *S. lilium*, their distribution was irregular, and they were clustered in groups located in the medial regions of the pyloric gland, seen in Figures 3A and B. In contrast, the distribution of GAS-immunoreactive cells in *A. cinerius* was more uniform, seen in Figure 3C. These endocrine cells were frequently oval in shape, illustrated in Figure 3D. They were distributed in the pyloric gland from the middle to apical portions, and they were more numerous in *A. cinerius* (126.9 ± 27.4/0.25 mm^2) than in *S. lilium* (75.8 ± 1.8/0.25 mm^2). They were also identified in the duodenal gland.

CCK immunoreactivity

CCK-immunoreactive cells were detected in both species of bat, distributed throughout the entire intestine. Their frequency was comparable in both species, and they were most abundant in the intestine III (14.2 ± 5.8/0.25 mm^2 in *A. cinerius* and 22.8 ± 6.5/0.25 mm^2 in *S. lilium*). They were detected in the intestinal glands and the epithelial components both species. Immunoreactive cells varied in shape from oval to piriform, as seen in Figures 4A and B. CCK-immunoreactive endocrine cells were not detected in the duodenal gland.

Glucagon immunoreactivity

Oval-shaped GLUC-immunoreactive cells were detected in the medial to basal glands of the fundic regions (0.91 ± 0.3) of *A. cinerius*, illustrated in Figure 5A. Their occurrence was low in the intestine and varied according to the species. In *A. cinerius,* their distribution was restricted to intestines II and III, and cells were most often oval in shape, seen in Figure 5B, while in *S. lilium* GLUC-immunoreactive were detected in all three intestinal regions.

Discussion

Recent advances in immunohistochemistry have made it possible to demonstrate the presence of a number of different types of endocrine cells in the GI tract of mammals. More than 15 different kinds of endocrine cells have been described in chiropteran GI tracts. Nine were described in the sanguivorous *Desmodus rotundus* (Yamada et al., 1984), eleven in the insectivorous *Pipistrellus abramusi* and *Plecotus auritus* (Yamada et al., 1988), four in *Molossus molossus* and *Lonchorhina aurita* (Santos et al., in press), eight in the nectarivorous and frugi-nectarivorous, *Anoura caudifer* and *Carollia perspicillata* (Ashihara et al., 1999), ten in the piscivorous *Noctilio leporinus* (Komori et al., 2000) and four in the pancreas of the frugivorous bat *Rousettus aegytiacus* (Michelmore et al., 1998). These studies showed inter-species differences and have suggested a correlation between endocrine cell distribution and feeding habits.

The present study mapped four types of endocrine cells in the GI tract of the frugivorous bats *A. cinerius* and *S. lilium*: endocrine cells immunoreactive for serotonin, GAS, CCK and GLUC. The general pattern of distribution of the endocrine cells in the GI tract of *A. cinerius* and *S. lilium* was similar to the findings reported in other mammalian species. However, when compared with other bats, the specific distribution of cells immunoreactive to GAS and CCK was wider, whereas the distribution of

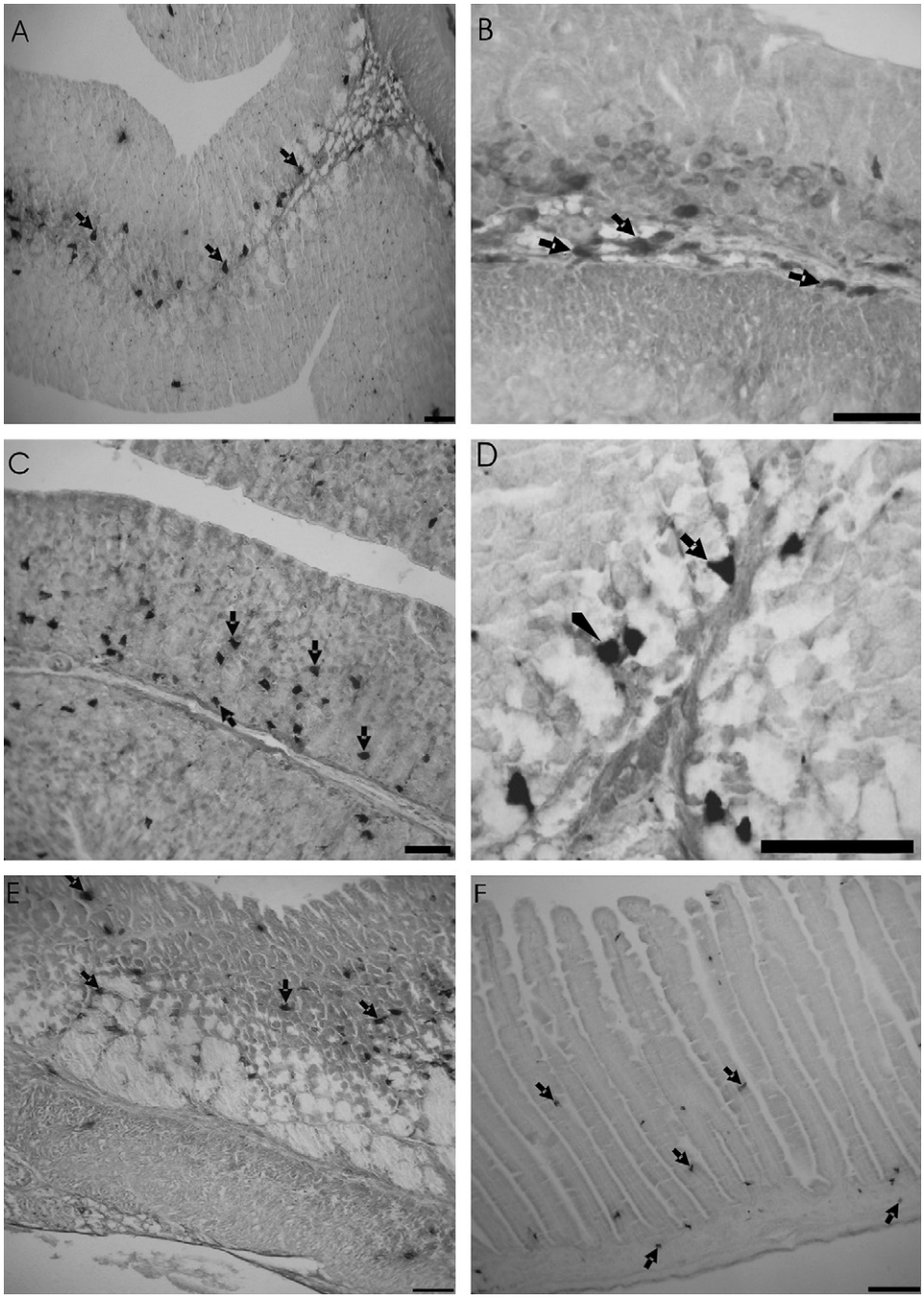

Figure 2. Serotonin/5-HT-immunoreactive cells. (A) Fundic glands of *A. cinerius*, serotonin/5-HT-immunoreactive cells in the basal half of the glands (arrows). Bar = 50 μm. (B) Pyloric gland of *A. cinerius* with serotonin/5-HT-immunoreactive cells in connective tissue in the submucosa (arrows). Bar = 50 μm. (C) Fundic glands of *S. lilium*; with serotonin/5-HT-immunoreactive cells distributed in the medial to basal region of the gland (arrows). Bar = 25 μm. (D) Fundic gland of *S. lilium* showing serotonin/5-HT-immunoreactive cells with oval (arrowhead) and triangular (arrow) cell shape. Bar = 50 μm. (E) Pyloric gland of *S. lilium* with serotonin/5-HT-immunoreactive cells in the apical and medial portion of the gland (arrows). Bar = 50 μm. (F) Intestine I of *A. cinerius* with serotonin/5-HT-immunoreactive cells in the intestinal epithelium and intestinal glands (arrows). Bar = 50 μm.

serotonin/5-HT- and enteroglucagon-immunoreactive cells was more restricted. It was interesting to localize the presence of CCK in all regions of the intestine of *A. cinerius* and *S. lilium*.

Serotonin (5-HT), a regulatory amine of mucosal enterochromaffin (EC) cells, plays an important role in the control of GI smooth muscle contraction and epithelial secretion (Fink et al., 2006). EC cells

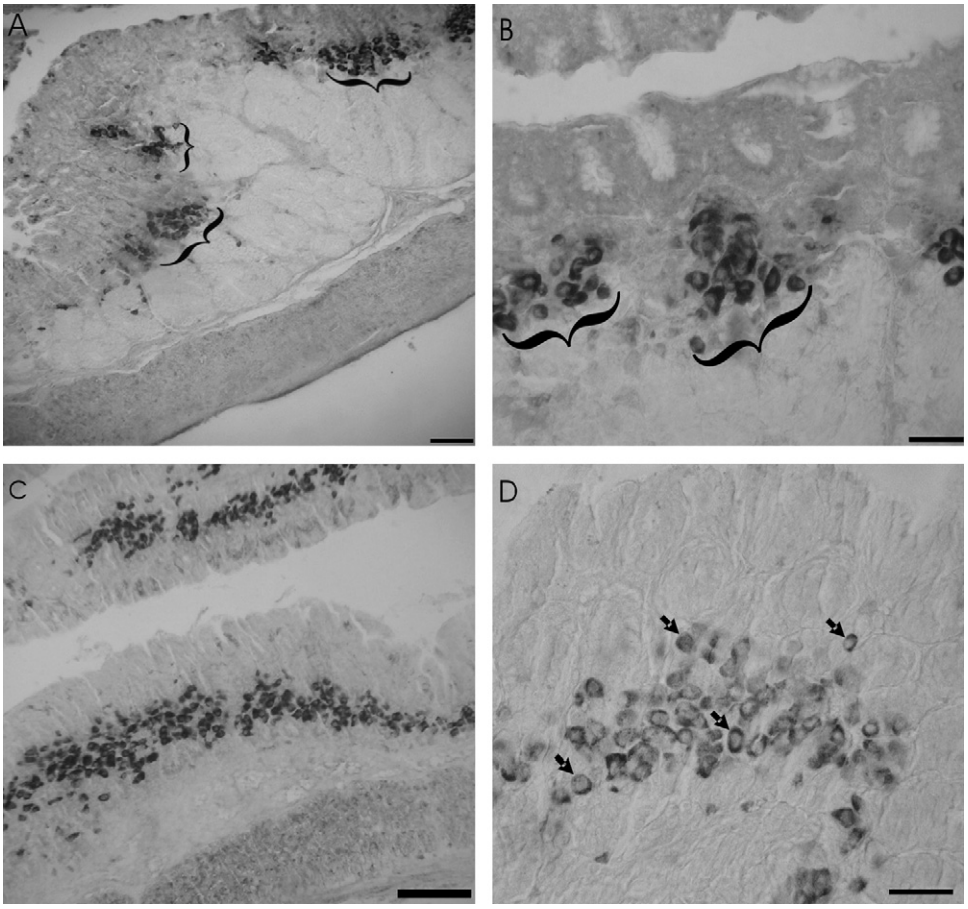

Figure 3. Gastrin-immunoreactive cells. (A) and (B) Pyloric gland of *S. lilium*. Note the distribution of gastrin-immunoreactive cells is irregular in the medial regions of the gland. Bar = 50 μm. (C) Pyloric gland of *A. cinerius*. Note the uniform distribution gastrin-immunoreactive cells. Bar = 50 μm. (D) Oval-shaped gastrin-immunoreactive cells in pyloric gland of *A. cinerius* (arrows). Bar = 50 μm.

constitute the largest endocrine cell population in the GI tract and produce over 90% of all the serotonin/5-HT synthesized in the body (Ahlman and Nilsson, 2001). Moderate numbers of serotonin/5-HT-immunoreactive cells were evenly distributed in all regions of the stomach and intestine, and showed similar distribution and frequency in both species of bats. This observation further demonstrates that serotonin/5-HT-IR cells have a wider distribution than other types of GI endocrine cells in the GIT of vertebrates. In *S. lilium,* as in the Phyllostomid bats *A. caudifer* and *C. perspicillata*, these cells were less abundant in the fundic glands. The abundance of serotonin/5-HT immunoreactive open type cells indicates that these cells probably secrete serotonin/5-HT by the paracrine pathway (Ashihara et al., 1999). These cells were also found in the duodenal gland of *S. lilium*, as has been reported in the insectivorous *P. abramus* (Yamada et al., 1988), *M. molossus* (Santos et al., in press), nectarivorous *A. caudifer*, frugi-nectarivorous *C. perspicillata* (Ashihara et al., 1999) and sanguivorous *D. rotundus* (Yamada et al., 1984), but these cells have not been identified in piscivorous *N. leporinus* (Komori et al., 2000), or in the frugivorous *A. cinerius* (Yamada et al., 1988).

GAS is a linear peptide hormone produced by G cells of the duodenum and in the pyloric antrum of the stomach (Tzaneva, 2003). GAS stimulates EC cells to release histamine. The increased histamine and the direct stimulation by GAS cause parietal cells to increase hydrochloric acid secretion in the stomach (Larsson, 2000). Previous investigations have revealed that in bats, GAS production might play some other roles within the pylorus itself (Mennone et al., 1986). The GAS-immunoreactive cells in this study were restricted in distribution to the middle to apical portions of the pyloric glands in both species of bat. The distribution pattern of the GAS-immunoreactive endocrine cells in the pyloric glands may facilitate a quick response to the luminal ingesta (Nisa et al., 2005). A remarkable difference in the distribution of GAS-immunoreactive cells is seen between bat species with

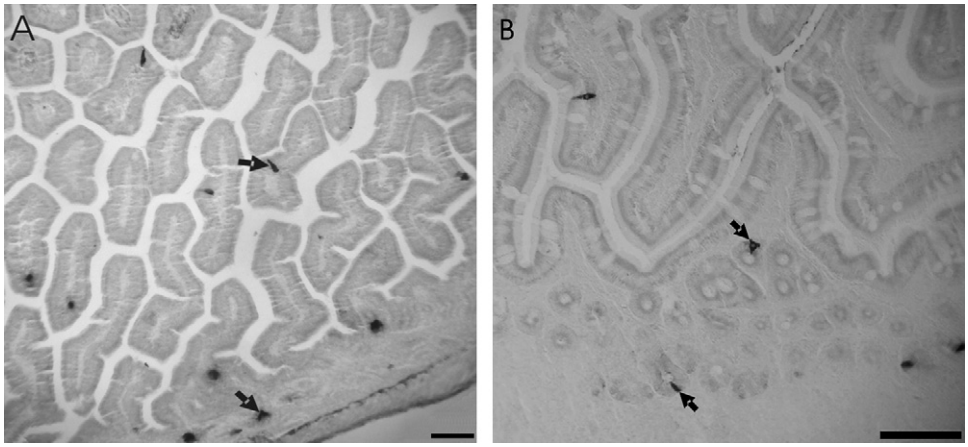

Figure 4. Cholecystokinin (CCK)-immunoreactive cells. (A) Intestine I of *A. cinerius*. Note that CCK-immunoreactive cells are dispersed in the interepithelial region and intestinal glands (arrows). Bar = 50 μm. (B) Intestine II of *S. lilium*, with CCK-immunoreactive cells in the intestinal glands (arrows). Bar = 50 μm.

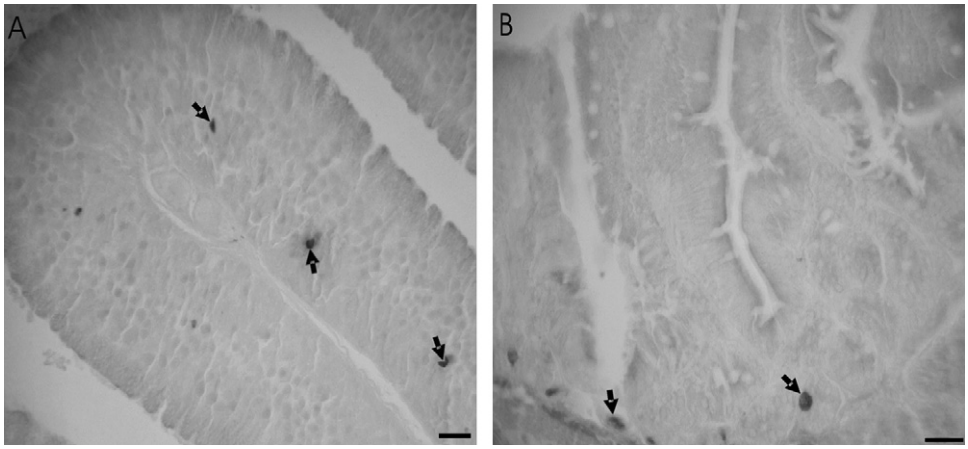

Figure 5. Enteroglucagon-immunoreactive cells. (A) Fundic region of *A. cinerius* with enteroglucagon-immunoreactive cells distributed in medial portions of the gland (arrows). Bar = 50 μm. (B) Intestine III of *A. cinerius*, with enteroglucagon-immunoreactive oval cells in the intestinal gland (arrows). Bar = 50 μm.

different feeding habits. In this study, the highest density of GAS-immunoreactive cells in the pylorus may be related to the frugivorous feeding habits of *S. lilium* and *A. cinerius*. Mennone et al. (1986) suggested that the GAS endocrine cells playing an important role in regulating the digestive function in frugivorous bats. These cells have been described in small numbers in the intestine of insectivorous (Yamada et al., 1988), piscivorous (Komori et al., 2000), frugi-nectarivorous and nectarivorous (Ashihara et al., 1999) bats. They were absent in the intestine samples examined in the present study.

CCK plays a key role in facilitating digestion within the small intestine. It is secreted from mucosal epithelial cells in the first segment of the small intestine (duodenum) and stimulates release of digestive enzymes from the pancreas and bile from the gallbladder into the small intestine. It is reported that CCK-like peptides are widely distributed among the lower vertebrates and these families of peptides were established early in animal evolution (Crim and Vigna, 1983). These CCK-immunoreactive cells were observed scattered along the entire length of the intestine of frugivorous bats studied here. A similar distribution has been reported in vampire (Yamada et al, 1984) and insectivorous bats (Yamada et al, 1988, Santos et al., in press). 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, *S. lilium*, *A. cinerius*, *A. caudifer*, *C. perspicillata* and *L. aurita* belong to the same

family of Phyllostomoidea and occasionally have similar feeding resources.

The GLUC gene is expressed along the GIT by highly specialized gut endocrine cells, designated L cells. The majority of L cells are classically thought to be located in the distal gut, predominantly in the ileum and colon. Their most reported action is to increase glycemia, counteracting the effects of insulin. GLUC-immunoreactive cells have been demonstrated in various mammals, and it is considered that the distribution pattern of these cells in the GIT of mammals show species-dependent variation (Ku et al., 2003). In the present study, the distribution of enteroglucagon-immunoreactive cells was similar to that of frugi-nectarivorous bats reported by Ashihara et al. (1999). 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).

In the study reported here, species-specific differences in the distribution of endocrine cells were observed along the gut of bats with similar feeding habits. However, the differences between these two species were less pronounced than those reported for bats with distinct feeding habits. Therefore, these data suggest a correlation between the distribution and frequency of endocrine cells and the feeding habits of these animals.

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