Immunohistochemical study of the six types of endocrine cells in the enteropancreatic system of the lizard *Tropidurus torquatus* (Squamata: Tropiduridae)

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(Received 5 January 2017; accepted 3 May 2017)

Abstract
The present study investigates the aspects of endocrine cells secreting cholecystokinin, gastrin, insulin, glucagon, somatostatin and serotonin in the enteropancreatic system of *Tropidurus torquatus*. The specimens were collected in Marambaia Island, Sepetiba Bay, Rio de Janeiro State, Brazil. The animals’ intestine and pancreas were removed, fixed, processed and then subjected to immunohistochemical techniques. Three cell types, immunoreactive (IR) to gastrin, cholecystokinin-8 and serotonin, were identified in the intestinal mucosa. Serotonin was found in the small and large intestines, whereas gastrin and cholecystokinin-8 cells were only observed in the large intestine of *T. torquatus*. Glucagon, somatostatin and insulin were not observed in the intestines at all. Only glucagon, somatostatin and insulin were identified in the pancreas of the studied lizards. The dorsal lobe showed a large number of endocrine cells scattered throughout the exocrine parenchyma and in the exocrine duct walls. The present study shows that the intestine and pancreas of *T. torquatus* hold most of the regulatory peptides, presenting a structure similar to that in other vertebrates such as birds and mammals. The endocrine cells identified in the lizards’ enteropancreatic system evidences the good phylogenetic preservation of the regulatory peptides’ molecular structure.

Keywords: Neuroendocrine system, immunolocalization, intestine, reptile, pancreas

Introduction
Reptiles are among the most ecologically and evolutionarily remarkable groups of living organisms since they have successfully colonized most of the planet, including oceans and some of the harshest and most environmentally unstable ecosystems on Earth (Pincheira-Donoso et al. 2013). Reptiles have accumulated a vast diversity of morphological, behavioral, ecological, life-history and defensive strategies to cope with the selective demands they have encountered due to exposure to hundreds of millions of years of radiation (Pianka & Vitt 2003; Pough et al. 2004; Fry et al. 2006). Apparently, reptiles are a useful future model – even more useful than other commonly used experimental animals such as rats, rabbits and pigs – for studies focusing on the physiological regulation of the digestive process, because of their good responses to feeding (Secor & Diamond 1998); therefore, they have become an important study object in different fields.

*Tropidurus* is one of the most characteristic genera occupying open environments in South America (Frost et al. 2001). The diversity of species belonging to this genus has grown over the years; recently the total number of species increased to 23 (Passos et al. 2011). This is one of the most broadly distributed lizard genera in South America, since the species in this genus are found from Northern Venezuela (and one locality by the Colombian border), Guyana, Suriname and French Guiana to Northern Argentina and Southern Uruguay. Their distribution covers almost the entire Brazilian territory and extends west to Bolivia and Paraguay (Carvalho 2013). *Tropidurus torquatus* (Wied-
Neuwied 1820) is a species of general habits, and opportunistic foraging of the “sit-and-wait” type, and it may change its habits depending on the environment. Although its diet is little restricted, studies show its eating preference for arthropods and, mostly, for vegetal contents (Teixeira & Giovanelli 1999; Siqueira et al. 2013; Vitt & Caldwell 2014).

Histological and immunohistochemical studies may help in understanding the species’ habits since the anatomy of reptiles’ gastrointestinal tract (GIT) changes according to order, studied species and foraging habitats. Many hormones, such as the regulator peptides, appear to act in the integration of the innumerable functions performed by the body; besides, they perform important regulatory actions in the physiological function of digestive tract cells (Fujita 1990; Larsson 2000; Huang & Wu 2005; Pereira et al. 2015). Moreover, secretory cells play an important role in lubricating the organ and protecting it from pro-teolytic degeneration and pathogenic microorganisms (Reid et al. 1988).

Several functions of different GIT segments are controlled by endocrine cells, forming a complex system able to secrete physiologically active polypeptide hormones and amines, which are disseminated among the epithelial components (Carvalheira et al. 1968). According to Deveney and Way (1983), the gastrointestinal hormones secreted by endocrine cells have important functions in the overall regulation of the digestive process, such as nutrient absorption, gut motility and intestinal blood flow. The endocrine cells in the lizard’s guts can vary in frequency and distribution depending on the species. Therefore, the aim of the present study was to determine the regional distribution and quantitative frequency of endocrine cells in the intestine and pancreas of *T. torquatus* through immunohistochemistry, as well as to improve the knowledge of the cell composition of the gastroenteropancreatic (GEP) system of reptiles.

Materials and methods

Collecting and preparing the biological material

Seven adult animals (four females and three males) were used in the experiment. They were captured in restingas found in the Marambaia peninsula (23°04’S, 43°53’W), Sepetiba Bay, Rio de Janeiro State, Brazil. The collected specimens were taken to the Histology and Embryology Laboratory of Rio de Janeiro Federal Rural University (UFRRJ) where they were sexed. The subjects were euthanized using 0.5% lidocaine, as recommended for reptile sacrifice in Resolution 714 from 2002, which was issued by the Federal Council of Veterinary Medicine. The current research was approved by the Ethics Research Committee of UFRRJ – process number 23083.012480/2010-64.

The animals’ coelomic cavities were opened, completely exposing the viscera, and intestine and pancreas were removed. The intestine was divided into small and large (Figure 1). The pancreas was divided into two lobes, the dorsal lobe along the small intestine, and the ventral lobe close to the spleen. The tissues were fixed in Bouin’s liquid for 6 hours and dehydrated through a graded series of ethanol solutions, and embedded in Histosec (Merk, Darmstadt, Germany) using routine protocols. Histological sections were cut with a rotary microtome to 5 μm thickness and mounted on glass slides pre-coated with 0.1% poly-l-lysine (Sigma-Aldrich, Inc. USA).

Immunohistochemical study

The sections for the immunohistochemical procedure were dewaxed and rehydrated according to the routine

![Figure 1. Anatomy of the gastroenteropancreatic system of *Tropidurus torquatus*. The esophagus, stomach, pancreas and intestine (small intestine and large intestine) are depicted.](image-url)
They were incubated in citrate buffer (pH 6.0–0.01 M) and placed in a microwave oven for 15 min, for antigen recovery. Subsequently, the sections were incubated in 3% H$_2$O$_2$ solution in methanol for 15 min to block any endogenous peroxidase. Next, they were incubated in 1:100 bovine serum albumin diluted (B4287; Sigma–Aldrich, Inc. USA) in phosphate-buffered saline (PBS) solution, for 30 min, in a humid chamber, at room temperature.

The intestine and pancreas sections were first incubated overnight at 4°C in the respective primary antibodies (Table I). Next, the sections were incubated in 1:200 “Universal” secondary antibody diluted at 1:200, for 30 min, and in avidin-biotin-peroxidase complex (ABC), diluted at 1:200, for 30 min (both from PK 6200, Vector Laboratories, Inc., USA). Subsequently, the peroxidase label was revealed through the reaction with Stable DAB/Plus (K 047, Diagnostic BioSystems, Inc., USA), which was prepared according to the instructions of the manufacturer. All dilutions and thorough washes, between stages, were performed using PBS (pH 7.4). The sections were counterstained in Harris’ hematoxylin, rinsed in deionized water, dehydrated through a series of ethanol and methylcyclohexane solutions, and mounted in Entellan (Merck & Co., USA) medium.

The polyclonal antisera produced by mammals were used because of the difficulty in obtaining T. torquatus antibodies against the studied proteins, and because these proteins are conserved at the phylogenetic scale (Huang & Wu 2005; Zhang & Wu 2009; Chandavar & Naik 2011; Hao et al. 2012). Negative and positive controls were used to investigate the specificity of the reactions. Bat intestine sections were used as positive controls, because they presented positive responses to these reactions in previous studies (e.g., Santos et al. 2008a, 2008b; Machado-Santos et al. 2009). The negative control was prepared by replacing the primary antibody with non-immune serum and PBS (pH 7.4).

Observation, photomicrography and cell counting

Figure 1 was made in a Leica M205C Stereomicroscope equipped with a photographic camera. The final photo is a result of the stacking process made with the software Leica Application Suite Version 4.5.

The intestine and pancreas photomicrographs from seven specimens were taken using a digital camera (Nikon Coolpix 4300) attached to the microscope (Olympus BX41). Ten fields per 10 (20× objective) intestinal mucosa, islet-like and exocrine region sections from each specimen were analyzed. The relative endocrine cell immunoreactive (IR) frequency was measured in a computerized image analyzer (Image-J software). The immunoreactive cell frequency was expressed as mean ± standard deviation (SD) per unit area (mm$^2$) of mucosa.

Results

Gastrin-, cholecystokinin-8- and serotonin (5-hydroxytryptamine)-IR cells were identified in the intestine of T. torquatus, but glucagon-, somatostatin- and insulin-IR cells were not observed there. Glucagon-, somatostatin- and insulin-IR cells were identified in the pancreas. Two different endocrine cell types were found in the enteropancreatic system. One cell type was classified as spindle-shaped (open-typed cells), because of its elongated spindle and pyriform shapes, as well as the narrow apex pointing toward the lumen. The other cell type was classified as spherical (closed-type cells or interstitial) due to its round and spherical features.

### Table I. Details of primary antibodies used in this study.

| Primary antibody | Code number | Working dilution | Source | Specificity |
|------------------|-------------|------------------|--------|-------------|
| Gastrin I        | G0785       | 1:1.000          | Sigma–Aldrich, Inc., USA | Specific for gastrin-containing gastrointestinal tract cells. It does not contain the C-terminal tetrapeptide commonly combined with CCK-8. |
| Cholecystokinin-8 (CCK-8) | C2581 | 1:8.000 | Sigma–Aldrich, Inc., USA | Specific for cholecystokinin-containing cells in the gastrointestinal tract. |
| Somatostatin     | A566        | 1:300            | Dako Corp., CA, USA | Specific for somatostatin-containing cells. |
| Glucagon         | G2654       | 1:2.000          | Sigma–Aldrich, Inc., USA | Specific against pancreatic glucagon, and cross-reacts with gut glucagon (enteroglucagon). |
| Insulin          | I2018       | 1:1.000          | Sigma–Aldrich, Inc., USA | Specific for insulin-containing cells. |
| Serotonin        | S5545       | 1:8.000          | Sigma–Aldrich, Inc., USA | Specific for 5-HT-containing cells. |
**Intestine**

The distribution patterns and relative frequencies of the endocrine cells in the intestine of *Tropidurus torquatus* are shown in Table II. Gastrin-IR cells were detected in the small intestine only of *T. torquatus* (Figure 2(a)–(c)). These IR cells were the second most predominant cell type in the intestine of the lizards studied herein (Table II). The gastrin-stained cells changed from spherical to spindle-shaped. The spindle-shaped gastrin-positive cells, which present relatively short cytoplasmic processes, were detected in the epithelia of the small intestine (Figure 2(b)). Spherical gastrin-positive cells were observed in the epithelial intestinal mucosa (Figure 2(c)).

**Pancreas**

The pancreas was divided into exocrine and islet regions. The distribution patterns and relative frequencies of the endocrine cells found in the pancreas of *Tropidurus torquatus* are shown in Table II. Somatostatin-IR cells were the second most predominant type in the pancreas of *T. torquatus* (Table II); they were found between the exocrine

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**Table II. Distribution and frequency of the endocrine cells of the intestine and pancreas of *Tropidurus torquatus* (mean ± standard deviation). NF = not found; CCK: cholecystokinin.**

| Antibodies | Intestine | Pancreas |
|------------|-----------|----------|
|            | Small     | Large    | Exocrine | Islets |
| Gastrin    | 28.3 ± 1.3| NF       | NF       | NF     |
| CCK        | 17.2 ± 3.2| NF       | NF       | NF     |
| Serotonin  | 46.2 ± 8.3| 18.5 ± 3.4| NF       | NF     |
| Somatostatin| NF       | NF       | 18.4 ± 2.2| 8.3 ± 3.6|
| Glucagon   | NF       | NF       | 26.1 ± 8.7| 11.2 ± 2.7|
| Insulin    | NF       | NF       | 9.2 ± 3.1 | 10.7 ± 2.2|

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**Figures**

Figure 2. Photomicrographs of gastrin-immunoreactive (IR) cells in the intestine of *Tropidurus torquatus*. (A–C) Small intestine. A, Gastrin-IR cells between the epithelial cells (arrows) (270×). B, Gastrin-IR cells of the epithelium of the intestinal folds, a few of the open type (arrow) and most of the closed type (740×). C, Highlighting the existence of closed cell types (arrows; 1000×).
acinar cells – as solitary cells – and, occasionally, in the pancreatic duct (Figure 5(a)). These IR cells were found in the peripheral parts of the islet regions and presented predominantly irregular shape (open-type cell) in the islet regions (Figure 5(b)), and elongated or oval shape...
(closed-type cells) in the parenchyma and in the exocrine duct walls (Figure 5(c)).

Glucagon-IR cells were the most predominant type in the pancreas of *T. toquatus* (Table II); they were located in the exocrine parenchyma, as single cells, or in two- to three-cell clusters between the exocrine acinar cells. All these IR cells were oval-shaped (closed-type cells) (Figure 6(b)) in the islet regions (Figure 6(a)).

Insulin-IR cells were the least abundant type in the pancreas of *T. toquatus* (Table II); they were found throughout the exocrine parenchyma, between the exocrine acinar cells – as solitary cells, or in three- to five-cell clusters (Figure 7(a)). These IR cells were located in the cell-cords (Figure 7(b)), in the islet-like regions; they were oval-shaped (closed-type cells) in the islet regions, and presented irregular shape (open-type cell) in the pancreatic parenchyma (Figure 7(c)).

**Discussion**

**Intestine**

Gastrin-IR, CCK-IR, serotonin-IR, somatostatin-IR, glucagon-IR and insulin-IR cells are six important
endocrine cell types found in vertebrates’ digestive tract. The regional distributions and relative frequencies of the endocrine cells found in the alimentary tracts can vary remarkably with animal species and feeding habits (Solcia et al. 1989; D’Este et al. 1994). The present study aimed at investigating the density of endocrine cells distributed in the enteropancreatic tract of Tropidurus torquatus.

The endocrine cells were divided into two types: round to spherical closed-type cells mainly located in the mucosal regions, and spherical to spindle-shaped open-type cells found in the epithelium (Ku et al. 2006). The closed-type cells assessed in the present study were mainly located in mucosal regions, whereas most of the open-type cells were found in the epithelial regions of T. torquatus.

Apparently, gastrin and CCK-8 come from the same ancestor, and a large fraction of these cells are seen in the human duodenum. They react with non-C terminal CCK antibodies and C-terminal gastrin/CCK antibodies, show immunoreactivity with C-terminal gastrin-34 antibodies and are co-localized with CCK in a varying portion of secretory granules (Solcia et al. 1989). Gastrin, which is secreted by G cells of the gastrointestinal tract, is the main physiological gastric acid secretion regulator (Solcia et al. 1987). It can also promote mucosal epithelial cell proliferation (Hernández et al. 2012), whereas the cholecystokinin secreted by endocrine intestinal mucosa cells (I cells) stimulates pancreatic enzyme secretion (Liddle 2000).

Gastrin-IR cells were restricted to the small intestine of T. torquatus in the present study, and are prevalent in the small intestine of reptile species such as the chelonians Chrysemys picta (Gapp et al. 1985), Testudo graeca, Mauremys caspica and Lacerta lepida (Perez-Tomas et al. 1989); the crocodilian Caiman latirostris (Yamada et al. 1987); and the lizards E. kingii (Arena et al. 1990), Podarcis hispanica (Burrell et al. 1992), T. volteri (Lee & Ku 2004), G. japonicus, E. chinensis, S. indicus and E. elegans (Huang & Wu 2005). However, the gastrin-IR cells were found in the large intestine of some reptilian species such as the Testudines, including Mauremys caspica (Tarakçi et al. 2005) and Trachemys scripta elegans (Zhang & Wu 2009).

Cholecystokinin-8-IR cells were only observed in the small intestine of T. torquatus, similarly to reptilian species such as the crocodilian C. latirostris (Yamada et al. 1987), and the lizards P. hispanica (Burrell et al. 1992) and T. volteri (Lee & Ku 2004). Some of the open-type cholecystokinin-8-IR cells in the intestine of T. torquatus present cytoplasmic processes that reach the adjacent cells possibly involved in paracrine secretion, as previously described by Chandra et al. (2010). However, most of the closed-type cholecystokinin-8-IR cells were found in the small intestine.

Figure 7. (A–C). Photomicrographs of insulin cells in the pancreas of Tropidurus torquatus. A, Insulin-immunoreactive (IR) cells are closed-type cells islet-like regions (arrowhead) and open-type cells at the exocrine parenchyma (arrow; 870×). B, View of insulin-IR cells in the islet-like regions (arrow; 500×). C, Insulin-IR cells in the pancreatic parenchyma (arrow; 740×).
Serotonin is a neurotransmitter synthesized by the serotonergic neurons of the central nervous system and by the endocrine cells in the GEP system (Rodrigues et al. 2005). This hormone has a strong effect on the regulation of digestive functions (Grundy 2008). The 5-HT could stimulate gastrointestinal mucosa secretion, smooth muscle contraction and the expansion of blood vessels, thus accelerating digestive tract movements (Wang et al. 2007). El-Salhy et al. (1985) reported that these IR cells are found in the digestive tract of all vertebrate species, a fact that suggests their establishment in this region at an early phase of vertebrate evolution.

Serotonin-IR cells were found in the intestine of T. torquatus and were the most predominant endocrine cells there. The same result was observed in different reptilian species, including in the crocodilians C. latirostris (Yamada et al. 1987) and Alligator sinensis (Wu et al. 1999); the chelonians M. caspica, T. gracea, L. lepida (Perez-Tomas et al. 1998); see also Taraçi et al. 2005 for M. caspica) and Ocadia sinensis (Liu et al. 2007); and the squamates E. kingii (Arena et al. 1990), C. chalcides, Z. madagascariensis (Morescalchi et al. 1997), T. woltleri (Lee & Ku 2004), G. japonicus, E. chinensis, S. indicus, E. elegans (Huang & Wu 2005), and T. scripta (Zhang & Wu 2009).

Most serotonin-IR cells presenting cytoplasmic processes reaching the intestinal lumen (open type) were observed in the intestine of T. torquatus, and this suggests that serotonin may be secreted through the exocrine pathway to the intestinal lumen. However, some of the closed types of these IR cells, reaching the adjacent cells, were also observed in the basal portion of the glands, thus indicating that these cells may secrete this hormone through the paracrine pathways. These different cell-signaling modes were previously described by Huang and Wu (2005) in the intestines of four squamates, namely G. japonicus, E. chinensis, S. indicus and E. elegans.

The somatostatin-, glucagon- and insulin-IR cells were not observed in any of the T. torquatus intestine regions. Lee et al. (1995) also reported the absence of somatostatin-IR cells in the GIT of the amur lizard. Glucagon-IR cells were not detected in the alimentary tract of the King’s skink species (Arena et al. 1990) or in the small intestine of the snakes (Masini 1986). However, these three IR cell types were found in the intestine of other lizard species, including L. lepida (Perez-Tomas et al. 1989), Chalcides chalcides, Zoonosaurus madagascariensis (Morescalchi et al. 1997) and T. woltleri (Lee & Ku 2004). The aforementioned studies suggest that the regional distribution of these cells changes among reptilians, and the present results seem to corroborate this pattern.

Pancreas

Somatostatin is secreted not only by the D cells of the pancreatic islets, but also by the hypothalamus and by certain intestinal cells; it also inhibits the secretion of other neuroendocrine hormones (Hsu & Crump 1989). The somatostatin cells scattered in the exocrine parenchyma, as well as the long protrusions exhibited by these cells, suggest paracrine functions performed by the zymogen cells. There are reports on somatostatin inhibiting the exocrine secretion by the pancreas (Raptis et al. 1978).

Somatostatin-IR cells were located in the exocrine pancreas and pancreatic duct, and around the periphery of cell-cords in the islet-like regions in the pancreas of T. torquatus. These IR cells were also located in the pancreas of different reptilian species such as A. carolinensis (Rhoten & Hall 1981), M. quinquetaeniata, U. aegyptia (El-Salhy & Grimelius 1981), C. ocellatus (El-Salhy et al. 1983), C. chalcides and Z. madagascariensis (Morescalchi et al. 1997); and in the Testudines G. picta (Gapp et al. 1985) and M. caspica (Ayala et al. 1989).

The glucagon secreted by A cells in the pancreas increases the blood glucose levels (Foa et al. 1957), whereas the insulin secreted by the B cells of the pancreas regulates the serum glucose levels (Hsu & Crump 1989).

The glucagon-IR and insulin-IR cells were observed in the islet-like regions and randomly located throughout the pancreatic parenchyma of T. torquatus. This result is similar to that previously reported for different reptilian species, including the squamates A. carolinensis (Rhoten & Hall 1981), M. quinquetaeniata, U. aegyptia (El-Salhy & Grimelius 1981), C. chalcides, Z. madagascariensis (Morescalchi et al. 1997), T. woltleri (Ku & Lee 2004) and E. carinata (Chandavar & Naik 2011); and the Testudines M. caspica (Ayala et al. 1989), T. scripta (Ku et al. 2001) and Melanochelys trijuga (Chandavar & Naik 2008).

The glucagon-IR and insulin-IR cell arrangements in the islets and isolated cells represent an endocrine network that may regulate the exocrine function in a paracrine fashion (Putti et al. 1992). Thus, the study showed that the glucagon and insulin cells do not necessarily need to form islets in all lizard species. Epple and Brinn (1987) concluded that the islets were scattered throughout the pancreas in turtles, crocodiles and in certain Squamata; actually, they are either individual or formed in smaller groups comprising two to four cells within the exocrine pancreas.

The density of endocrine cell distribution is related to the species’ feeding habits, food components and living environment. The morphologies of endocrine cells are in compliance with the endocrine...
and exocrine functions (Zhita et al. 2011). Therefore, the different endocrine cell types identified in the enteropancreatic system of *T. torquatus* are similar to those found in other reptiles and other vertebrate species, which is a characteristic of vertebrate phylogeny.

**Acknowledgements**

The authors thank I. L. C. Meirelles for her technical assistance, the Rio de Janeiro State Research Foundation (FAPERJ) for financial support and UFRRJ for the exchange of Program Internal Exchange Scientific Initiation – PROIC granted to the first author. The research was approved by the Ethics Research Committee of the Federal Rural University of Rio de Janeiro, Seropédica, Brazil (process number 23083.012480/2010-64). The authors thank H. R. Silva for help in the animals’ collection. License number for wild animal capture: SISBIO 506961. No conflict of interest is reported.

**Funding**

This work was supported by the Rio de Janeiro State Research Foundation (FAPERJ); and UFRRJ for the exchange of Program Internal Exchange Scientific Initiation.

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