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Localization of glucagon and insulin cells and its variation with respect to physiological events in *Eutropis carinata*

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Abstract. The aim of the present investigation was to localize glucagon and insulin immunoreactive (IR) cells of pancreas during annual seasonal cycle of reproduction and to find out whether they had any effect on the regulation of plasma glucose level in the skink *Eutropis carinata*. Immunolocalized pancreatic cells revealed significantly different mean numbers in different periods of reproduction. The numbers of glucagon-IR and insulin-IR cells were highest in recrudescent period which was corresponded with low plasma glucose level. Unlike other lizards the arrangement of insulin cells in the central core and glucagon cells at the periphery was absent instead glucagon-IR and insulin-IR cells were paracrine in arrangement. Among the two immunoreactive cells glucagon-IR cells were predominant. Morphological differences between two cell types were observed by electron microscopy after staining with uranyl acetate and lead citrate. Plasma glucose showed cyclic change being highest during reproductive period.

Keywords. Glucagon-IR, Insulin-IR, Lizard, Plasma glucose, Reproductive cycle.

INTRODUCTION

Eutropis (Mabuya) carinata is an insectivorous skink (lizard), inhabitant of South Asia. It runs swiftly, usually basks during winter and shifts to shady area in summer. El-Salhy and Grimelius (1981) made histological and immunohistochemical investigation of endocrine pancreas of the grass lizard, *Mabuya quinquetaenia-ta*, and that of the desert lizard, *Uromastyx aegyptia.* Rhoten and Hall (1982) examined the differentiation of islets of Langerhans in the lizard *Anolis carolinensis.* The endocrine pancreas of the lizard, *Podarcis hispanica,* consists of single scattered cells or small groups of two to five cells forming islet-like structures (Lopez et al., 1988) and that of *Podarcis s. sicula* is concentrated more in the splenic than in duodenal region and never formed large clusters (Putti

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et al., 1991). The comparative morphology of islets of Langerhans in 11 species of lacertids demonstrated the central core of B cells and A cells at the periphery with the predominance of earlier-cells (Putti et al., 1992). Della Rosa and Putti (1995) reported the distribution and frequency of different endocrine cells in the lacertid pancreas. Ku and Lee (2004) studied the regional distribution and frequency of the pancreatic endocrine cells in the splenic lobe of the grass lizard, *Takydromus wolteri*, by immunohistochemistry.

Most of the earlier studies on endocrine pancreas of different lizards were confined to identifying different cell types by histology, immunocytochemistry and electron microscopy where as the present investigation was under taken to localize glucagon and insulin cells of pancreas during annual seasonal cycle of reproduction and to find out whether they have any effect on the regulation of plasma glucose level in the skink *E. carinata*.

MATERIALS AND METHODS

Animals

We collected *E. carinata* from Manasagangotri campus, Mysore (Latitude 12°18'N; Longitude, 76°42'E; Altitude, 777 m asl). They were maintained in the reptile house in the open and fed silk moth (*Bombyx mori*) ad-libitum. "Guidelines for Care and Use of Animals in Scientific Research" were followed (Anonymous, 2000). Experimental protocols were approved from Institutional Animal Ethics Committee (IAEC). The animals were studied in the annual seasonal cycle of reproduction (2007 to 2009) which is distinguished into three separate periods namely regenerative, reproductive and recrudescent. Ten adults of *E. carinata* weighing 12-30 g were utilized in each period irrespective of the sex. As the animals were collected from the field it was not possible to determine their age, hence measurements were taken. The animals were injected with sodium pentobarbital (50 mg/kg body weight) intra peritoneal for recording their body length and weight and were sacrificed. The length of the animal was measured from tip of the snout to tip of the tail. The pancreas was freed, its length measured and weighed and fixed in Bouin-Hollande sublimate solution for 18-20 h and processed for light microscopy and immunocytochemistry.

Plasma glucose

Simultaneously, blood samples from carotid artery were collected and centrifuged at 4 °C and 10,000 rpm for 10 minutes. The separated plasma of ten active animals (10 consecutive samples from each animal) was immediately used for estimation of glucose by enzyme glucose oxidase method of Trinder (1969) as described earlier (Chandavar and Naik, 2004; 2008).

Histology

Paraffin embedded pancreas from each of the animals was sectioned at 4-5 μm in series. Ten to 15 sections were mounted on a slide and every second slide was used for light microscopy. Chrome alum Hematoxylin and Phloxin (CHP) staining method (Gomori, 1941) was employed for light microscopy. Sections were treated with acidified $KMnO_4$ and subsequently decolourised with sodium bisulphite, stained with hematoxyline, differentiated in 1% acidified water, counter stained in phloxin for few minutes and mordant in phosphotungstic acid. CHP stained sections were used

for islet measurement at their longest axis at 400×. The size of islet was calculated by random selection of 100 observations in each period (Weesner, 1960; Ku and Lee, 2004), using software Image pro express, version 5.1.

Immunocytochemistry

All the chemicals used in immunocytochemistry were purchased from Sigma-Aldrich, USA. Every third slide was used for glucagon cell localization and the fourth slide having sections of the same islet was used for insulin cell localization. They were immunolocalized by the ExtrAvidin-Biotin Peroxidase method after Yang et al. (1999) and as per the instruction manual provided with the kit. The paraffin embedded sections were deparaffinised processed through grades of alcohol, washed in running water, pretreated with 3% H_2O_2 in methanol, rinsed with phosphate buffered saline (PBS, pH 7.6) and non-specific reactive sites blocked with 5% normal goat serum. They were then incubated for 1 h at 37 °C in a humidified chamber with the respective primary monoclonal mouse antibody (porcine glucagon was used as immunogen, product no G2654; diluted 1:2000 and human insulin was used as immunogen, product no I2018; diluted 1:1000). The sections were carefully washed 10 to 15 times with PBS and incubated for 30 min with biotinylated goat anti-mouse immunoglobulin secondary antibody and extravidin-peroxidase (Mouse extravidin peroxidase staining kit Stock No. EXTRA-2, Sigma), each diluted 1:20. PBS with 5% normal goat serum was used as diluent. The peroxidase activity was demonstrated using 0.7 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.17 mg/ml urea hydrogen peroxide and 0.06 M Tris buffer for 1-3 min. To show that the labeling is specifically due to the primary antibody, the primary antibody is replaced with similarly diluted normal serum from the same species, keeping all the other steps the same in controls (Burry, 2000). Another control for specificity which included omission of primary monoclonal antibody and parallel incubation with antibody reabsorbed with excess of respective antigen. No immunostaining was obtained in the controls. This further confirms that the immunolocalization has taken place in islets only.

Pancreatic sections containing islets were observed throughout the pancreas. Immunoreactive cell count was done by random selection of 100 sections in every period. Glucagon-IR and insulin-IR cell counting was done separately by using software Image pro express, version 5.1.Total number of glucagon-IR and insulin-IR cells of all ten animals in every period was considered as 100 percent and quantitative analysis in terms of percentage of glucagon-IR and insulin-IR cells was calculated. Digital photographs were taken using Olympus B × 60.

Liver histochemistry

Specimens autopsied to collect blood and pancreases were also used for histochemical localization of liver glycogen. Liver was fixed in Rossman's fixative and then processed, sectioned at 9 -10 μm and stained following Periodic Acid-Schiff (PAS) technique of Hotchkiss (1968). The PAS positive masses localized in the cytoplasm was taken into consideration for qualitative analysis of glycogen.

Electron microscopy

Pancreases of 0.5 mm^3 were fixed for 24 h at 4 °C in 3% glutaraldehyde in 0.1 M phosphate (pH 7.2-7.4), then post fixed in 1% buffered Osmium tetroxide, en-bloc stained with 2% uranyl acetate in 95% ethanol and embedded in Araldite-Cx resin after polymerizing it at 60 °C for 48 h. Ultrathin sections were obtained with LKB Ultracut microtome, stained with uranyl acetate fallowed by lead citrate, and examined by FM Jeol, EM, electron microscope (Johannessen, 1978).

Statistical Analysis

The measurements were expressed as mean ± SD for islet size (in mm); cell count (number) and plasma glucose level (mg/dl) during different periods was carried out using analysis of variance (ANOVA). Wherever the ANOVA values (F) were found to be significant, Duncan's Multiple Range Test (DMRT) was applied.

RESULTS

Initiation of gonad activity occurred during August-September (Table 1) in both the sexes of *E. carinata* and this duration is designated as regenerative period, which corresponded with late monsoon. Peak of gonad activity was observed during October-December, during which the animals exhibit well developed ova/ testis. This is referred to as reproductive period. Testis in males become smaller; oviduct of females had either fully mature eggs in them which was about to lay or they had no eggs with reduced oviducts in recrudescent period which fall in the months of January-July. Different periods of reproductive cycles were assigned by careful observation of the status of the gonad during two successive cycles of reproduction.

Weight of the animals varied with the season, being highest in regenerative period (Table 2). The mean pancreas weight was least in this period while the length of the pancreas was moderate. The reproductive animals weighed minimum, having a higher pancreas weight. During recrudescence, the animals weighed moderate. Significant difference existed between periods with respect to weight of the animal (Table 2). The mean weight of pancreas during reproductive period was higher and differed significantly from the other two periods. The pancreas on an average measured 2.97 ± 1.03 cm in length and weighed 0.18 ± 0.16 g. Animal length and pancreas length did not differ significantly between the periods.

The islets in *E. carinata* were irregular without connective tissue capsule. They stained as dark blue clumps of cells surrounded by lighter stained exocrine pancreas in CHP method, unlike in laboratory mammals (rat). Conspicuous variations in staining property of islets between *E. carinata* and mammals reveal that the cytoplasmic granules of glucagon and insulin cells differ from that of mammals.The insulin cells stained darker, round or elongated with central nuclei but the glucagon cells were not evident in CHP method. Hence, immunocytochemistry was carried out to localize both the cell types. The immunoreactive cells were located in the exocrine pancreas as solitary or two to three cell clusters or as islet throughout the gland (Fig.1, A to F). Smaller clusters were not evident in CHP method. Larger islets exhibit capillary spaces in them and were oriented towards the blood vessel, indicating their endocrine property. This was further confirmed by electron microscopy (EM) (Fig. 2, A and B).The endocrine pancreatic cells were found to be distributed along the capillaries. Morphological differences between two cell types were observed. Under EM the glucagon cells were oval in shape. Their nuclei were placed away from the centre with or without eccentric nucleoli. Cytoplasm showed the presence of dense granules. The secretory granules were round, electron dense and devoid of electron lucent space (Fig. 2B). Insulin were elongated or oval in shape with central round nuclei. Secretory granules were uniformly distributed throughout the cytoplasm. The granules were characteristically

Table 1. Annual seasonal cycle and reproductive events in *E. carinata.*

		Reproductive periods		
		Regenerative	Reproductive	Recrudescent
Gonad activity		Initiation	Peak	Regression
Month		Aug-Sept	Oct-Dec	Jan-July
Season		Late monsoon	Winter	Summer/monsoon
Temperature:	Max	31 ± 2 °C	28 ± 0.5 °C	37 ± 2 °C
	Min	19 ± 0.5 °C	13 ± 2.1 °C	18 ± 2 °C

Note: Specimens were collected at different periods of the year. Autopsy was carried out in the above mentioned months and temperature was recorded during those months.

Table 2. Different periods of reproduction and morphometric measurements in *E. carinata.*

	Reproductive periods			Statistics	
	Regenerative	Reproductive	Recrudescent		
Parameters	(mean ± SD)	(mean ± SD)	(mean ± SD)	F-value	P-value
Animal weight (g)	31.26 ± 5.10	17.4 ± 4.85	22 ± 3.76	23.494	< 0.001
Animal length (cm)	26.80 ± 3.32	27.5 ± 1.58	25.1 ± 1.65	2.817	0.077
Pancreas weight (g)	0.039 ±.016	0.46 ± 0.44	0.052 ± 0.008	8.865	0.001
Pancreas length (cm)	3.48 ± 1.3	2.75 ± 1.31	2.7 ± 0.54	1.545	0.232
Plasma glucose (mg%)	205 ± 57	233 ± 47	164 ± 25	17.645	< 0.001
Abdominal fat	+++	-/+	++		
Liver glycogen	++	-/+	+++		

Note: Mean with same letters is not significantly different from each other. SD, standard deviation; +++, maximum; ++, moderate; -/+, minimum.

membrane bound, filled with dense, rectangular or polymorphic matrix. A distinct electron lucent space between membrane and matrix was prominent (Fig. 2B). There was no significant change in the distribution of islets throughout the pancreas in both the sexes.

During regenerative period, the islets measured 0.22 ± 0.09 mm with lesser count of both glucagon-IR (2645 ± 32) and insulin-IR cells (2164 ± 24). The plasma glucose was moderate. The regenerative animals exhibited very few PAS positive masses in their liver samples (Table 2).

The animals were found basking during reproductive period and it corresponded with winter. The pancreas showed the presence of islets in smaller clumps. On an average, the islet measured 0.41 ± 0.24 mm. Glucagon-IR (6624 ± 35) and insulin-IR cell (5425 ± 36) number was higher than that during regenerative period. The plasma glucose recorded the highest value. There was no localization of PAS positive masses in liver sections in this period (Table 2).

The maximum number of glucagon-IR (9351 ± 36) and insulin-IR (7651 ± 40) cells was evident during recrudescence. The size of the islet measured 0.69 ± 0.26 mm. The

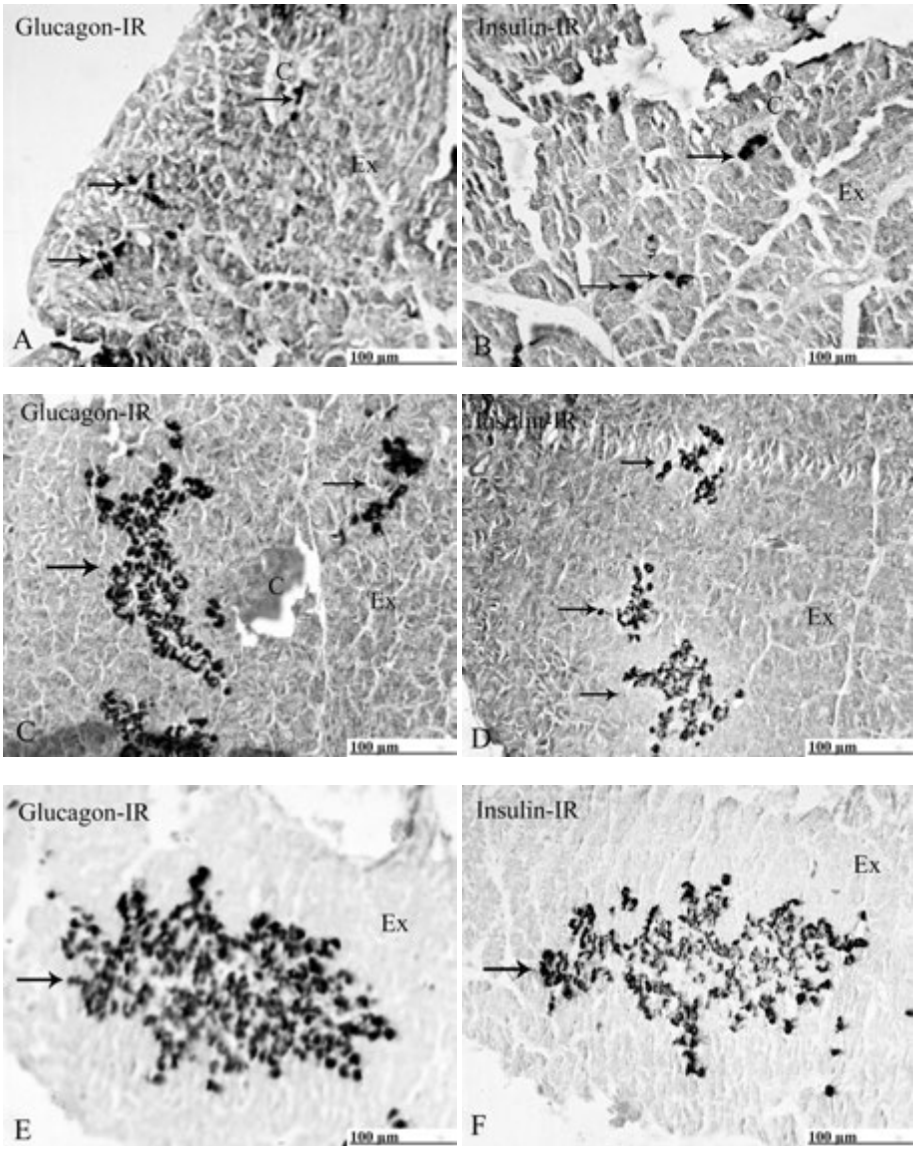

Fig. 1. Left top (A). Pancreas of regenerative period showing very few glucagon-immunoreactive cells appearing dark (arrows) between acini (Ex), blood capillary (C) is also seen. × 200. Right top (B). Succeeding section of regenerative period showing very few insulin- immunoreactive cells appearing dark (arrows) between acini (Ex) and near the blood capillary (C). × 200. Middle left (C). Pancreas of reproductive period showing glucagon-immunoreactive cells in larger group as dark mass (arrows) between acini (Ex) and blood capillary (C). × 200. Middle right (D). Succeeding section of reproductive period showing darkly stained larger group of insulin-immunoreactive cells (arrows) than the earlier period between acini (Ex). × 200. Bottom left (E). Pancreas of recrudescent period localized for glucagon- immunoreactive cells which appear as dark mass between acini (Ex). × 200. Bottom right (F). Succeeding section of recrudescent period showing darkly stained insulin- immunoreactive cells (arrow) between acini (Ex). × 200. All the above photographs are the representatives of each period.

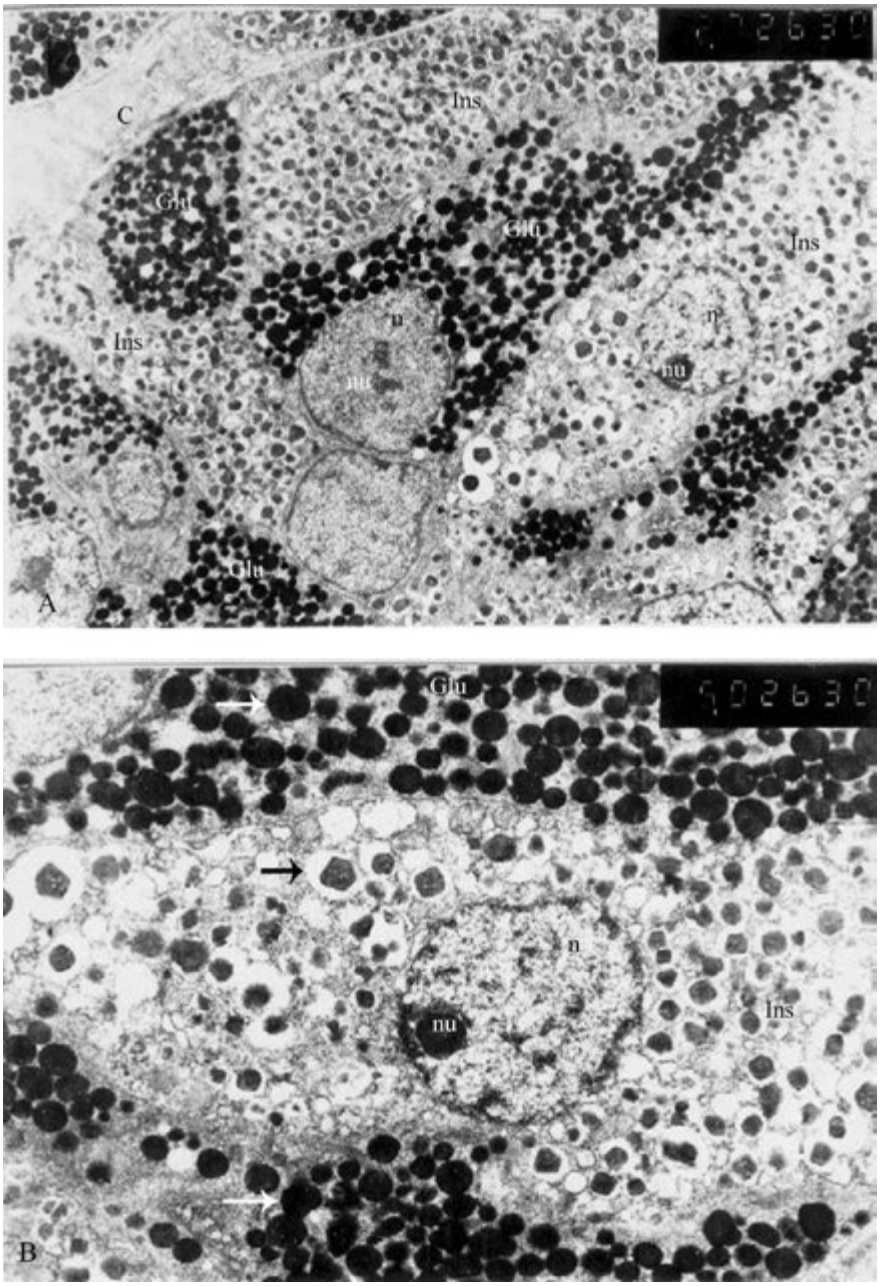

Fig. 2. (A) Electron micrograph of islet region showing glucagon (Glu) cell with basal nucleus (n) and insulin (Ins) cell with central nucleus (n) and eccentric nucleolus (nu). The endocrine cells were surrounded by the blood capillary (C). × 5400. (B) Electron micrograph of an enlarged region of above islet showing glucagon (Glu) and insulin (Ins) cell with nucleus (n) and nucleolus (nu) × 10000. Secretory granules of glucagon cells were electron dense (white arrow) with closely fitting membrane. Secretory granules in insulin cell were prominent with electron lucent space (black arrow) between membrane and matrix.

plasma glucose was lowest of all the periods. Liver sections of recrudescent period reveled intensely stained PAS positive masses.

The mean cell count for both-IR cells during recrudescence was highest and differed significantly from regenerative and reproductive periods. Immunolocalized pancreatic cells revealed significantly different mean numbers of localized cells in different phases of reproduction being highest in recrudescence. But proportion of glucagon-IR (55%) and insulin-IR (45%) cells remained similar in all periods with paracrine arrangement being adjacent to one another. This was also evident under electron microscopic studies (Fig. 2A). Significant difference in mean plasma glucose level between periods was observed being highest in reproductive period. Mean size of the islet also showed significant variation between periods being highest in recrudescent period.

DISCUSSION

The food consumed by the animals during regenerative period was converted and stored as reserve food. Conversion of food into fat and muscle mass rendered the animals to weigh higher. Reproductive period appears to be energetically expensive and are found to be reliant on stored energy in *E. carinata*. Therefore the animals of this period had no abdominal fat and glycogen mass in their liver. Absence of reserve food (glycogen and fat) may be due to higher cell count of glucagon-IR and insulin-IR cells in comparison to regenerative period. The food consumed by the animals was mainly utilized for the development and maturity of gonads. The reserved food was found diverted to reproduction instead of constructing the body mass, and the animals, on an average weighed the least of all the periods though they were gravid. Decreased liver glycogen may be one of the contributing factors for increasing the plasma glucose during reproductive period which is accompanied by increased glucagon cells rendering glycogenolysis compared to regenerative period. The phosphorolysis of glycogen is mainly mediated by glucagon (Bollen et al., 1998).

Glucagon-IR and insulin-IR cells increased from reproductive period to recrudescent period. The increase in number of both cell types may be due to stimulus of higher plasma glucose of reproductive period. Increased insulin cells of this period than the preceding periods contributed gradually in building up of liver glycogen and this accounted for decrease in glucose output. The conversion of glucose into glycogen in liver and availability of glucose to peripheral tissues accompanied by endocrine cells resulted in building up of muscle mass. Increased insulin-IR cells facilitated glucose uptake by peripheral tissue as well as anabolic effect on liver to construct glycogen. As a result the animals weighed more than those in reproductive period with least plasma glucose. The regulation of hepatic glucose metabolism has a key role in whole-body energy metabolism, as the liver is able to store and to produce glucose (Foufelle and Ferré, 2002). Glycogen is stored as a reserve of glucose in liver for extra hepatic tissues. Another glycogenic stimulus for the liver is insulin. As glucagon-IR cells were more numerous than insulin-IR cells, stored energy in the form of abdominal fat were utilized. *Eutropis carinata* exhibits annual cycle of energy storage in the form of abdominal fat.

Due to cyclic change in plasma glucose, liver glycogen, abdominal fat, glucagon-IR

and insulin-IR cell count, *E. carinata* exhibited significant difference in body weight and pancreas weight and not in their lengths, which usually remains static for a species.

In the present investigation monoclonal antibodies were used to localize glucagon-IR and insulin-IR cells. Use of monoclonal antibodies is the most reliable method for localizing glucagon and insulin cells in pancreas. The most abundant endocrine cell type was glucagon-IR cells (55%). In most lacertids studied, the central core consisted of B cells and A-cells at the periphery, with predominance of B-cells (Putti et al., 1992; El-Salhy et al., 1983). In the pancreas of the reptilian species, insulin-IR cells were present as solitary cells or in groups. They were located in the central core of the pancreatic islets and the most predominant cell type (Perez-Tomas et al., 1989; Morescalchi et al., 1997). In the present study of *E. carinata*, very few glucagon-IR and insulin-IR cells were scattered or present in smaller groups, particularly in regenerative period. In larger islets, glucagon-IR and insulin-IR cells were found scattered throughout the islet. This was further confirmed by EM. Insulin containing B cells or glucagon containing A cells were not clustered in the islet as in other lizards (El-Sahly et al., 1983; Perez-Tomas et al., 1989; Putti et al., 1992; Morescalchi et al., 1997), but showed paracrine association with one another. Paracrine interactions might have increased both cell types in number form regenerative to reproductive and from reproductive to recrudescent period. Among the two cell types glucagon-IR cells were predominant (55%) in all the periods. In this respect, *E. carinata* appears to be unique.

The normal fasting blood glucose level of five lizard species namely *Eumeces obsolecte*, *E. skiltonianus, E. fasciatces, A. carrolinensis*, and *Sceloporus accidentalis* ranged 74.0-113.9 mg% as against post-pranadial blood glucose level of 142.5-219.7 mg% (Miller and Wurster, 1956). The highest mean plasma glucose in *E. carinata* was 233 mg%. This indicates that the lizards are capable of tolerating values exceeding 200 mg/dl. In the present investigation it was found that physiological events were accompanied by substantial fluctuations in plasma glucose. In *E. carinata,* the study leads us to suggest that glucagon and insulin cells could regulate fat, glycogen and glucose metabolism in paracrine manner on reproductive events.

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