Variation in plasma glucose and pancreatic β cells in the turtle, *Lissemys punctata* (order: Chelonia; family: Trionychidae)

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Abstract

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The present study relates to the determination of the plasma glucose level and volumetric analysis of β cells in pancreatic islets of the soft-shelled turtle *Lissemys punctata* during different phases of its reproductive cycle. Reproductive events play a vital role in influencing the plasma glucose level and β-cell behaviour in the pancreatic islets. The colour of the pancreas is either yellowish or pinkish, depending on endocrine activity. Islets are present throughout the gland and range from individual cells to small or large clumps, depending on the seasonal cycle. Splenic islets are dense with more blood capillaries and nerve innervations irrespective of sex and season. The endocrine cell mass forms irregular patches without connective tissue capsule. β cells occupy the inner region of the islets, being surrounded by other cell types. *Lissemys punctata* exhibits higher β-cell activity during hibernation. Most insulin-secreting cells acquire a larger size during the regressive period. An analysis indicates that β cells outnumber the non-β endocrine cell mass in both number and per cent volume. There is negative correlation between islet mass and animal weight. Between the periods of reproductive cycles, a difference exists with respect to fasting plasma glucose and β-cell volume.

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Introduction

Plasma makes up 60–80% of blood volume and consists of a variety of substances in reptiles as in other vertebrates. Dessauer (1970) reports that the concentration of glucose in blood varies with the season in many reptiles, such as testudians, squamata and crocodilia. Daily events as well as seasonal metabolic cycles are observed to influence blood glucose in reptiles. Remarkably little knowledge is available with reference to fasting plasma glucose levels and activities of the pancreatic islet cells in untreated reptiles. Miller and Wurster (1956) have studied the relationship between plasma glucose and the pancreatic islets of normal and treated lizards. However, no study has been carried out on turtles in this regard. Correa *et al*. (1960) investigated the fate of β cells during oral administration of glucose in the turtle *Chrysemys dorbigini*. Later, Muniz and Marques (1989) in the same species found qualitative changes in the endocrine pancreas from hyperglycaemic streptozotocin treatment. Recently, Ray and Maiti (2001) reported a positive correlation between ambient temperature and blood glucose in *Lissemys punctata*. The blood plasma of turtles contains glucose in moderate concentrations while in lizards the glucose level is consistently higher than in other reptiles (Dessauer 1970; Chandavar 2002). Physiological events are accompanied by substantial fluctuations in plasma glucose. Glucose regulation is dependent on the β-cell volume in all major groups of vertebrates. The objective of the present investigation is to understand the correlation between these parameters in *L. punctata*.

Materials and Methods

The winter-hibernating Indian turtle *L. punctata* exhibits adaptation to a variety of environments. They remain concealed in loose soil or in water during hibernation. After hibernation, they become voracious eaters and hyperactive.

Table 1 Ambient temperature, seasonal cycle and reproductive events in *Lissemys punctata*

Parameter	Reproductive periods		
	Regenerative	Reproductive	Regressive
Months	March–June	July–August	September–February
Seasons	Summer	Monsoon	Winter
Temperature range	37–19 °C	28–19 °C	28–11 °C
Reproductive stage	Initiation of gonadal activity	Well developed sperms and ova Mating occurred	Regression of gonads
Activity of animals	Hyperactive	Active and aggressive	Sluggish, hibernating

Their sex ratio is maintained at 1 : 1 (Das 1995). Singh (1974, 1977) reported the reproductive cycle of *L. punctata*. On average, the animals weigh between 900 and 2400 g.

Adult animals of both sexes were collected from their natural habitat, Kabini reservoir (latitude 11°0′30″N; longitude 76°21′12″E; catchment area 2142 km², about 60 km away from Mysore) and were maintained in an outdoor cement tank containing water and sand. The cistern measured $7 \times 5 \times 5$ feet ($2.1 \times 1.5 \times 1.5$ m) with a raised bottom at one end for basking. The animals were fed fresh carrot and spinach *ad libitum*, but were not fed 48 h prior to autopsy. During experimentation the ambient temperature of Mysore varied with the season (11–37 °C). The reproductive events in *L. punctata* differed with respect to season (Table 1).

Four animals of *L. punctata* were used in each season. They were anaesthetized with chloroform and their body measurements and weights were recorded. They were killed by decapitation and blood samples were collected from the carotid artery. The blood samples were centrifuged at 4 °C at 6622 *g* for 10 min. The separated serum was used for estimation of plasma glucose by the enzyme glucose oxidase method. The estimation was done using Dr Reddy's GOD/POD Kit (Plummer 1987) and Spectrophotometer, BIO-TEK Instruments, INC (USA) Model μQuant. The glucose kit is based on Trinders's method in which glucose oxidase (GOD) and peroxidase (POD) enzymes are used along with the chromogen 4-aminoantipyrine and phenol. The enzyme GOD gives D-gluconic acid and hydrogen peroxide oxidizes glucose. Hydrogen peroxide in the presence of the enzyme POD oxidizes phenol, which combines with 4-aminoantipyrine to produce a red-coloured quinone-imine dye. Plasma glucose was determined for all the seasons by averaging the values of multiple samples from active animals maintained at room temperature under fasting conditions for 48 h.

Meanwhile, the pancreas was removed, fixed in Bouin–Hollande solution, processed and sectioned (4-μm) for light microscopy. Every fifth oxidized slide was stained with chrome alum haematoxylin phloxin (CHP) and phosphotungstic acid was used as a mordant after acidification, slightly modified after Gomori (1941). β-cell count was performed at regular intervals throughout the pancreas. Only those islet cells in which the nucleus was visible were counted using the blood cell counter. The density of β cells was calculated with respect to non-β-cell mass during each period. The size of islets, and β cells was calculated by random selection of at least 100 observations for each season.

Statistical analysis of β-cell count, β-cell density and per cent plasma glucose level for different periods was carried out using analysis of variance (ANOVA). Wherever the variance value was found to be significant at 5%, Duncan's Multiple Range Test (DMRT) was applied. Statistical presentation was organized using STATISTICAL PRESENTATION SYSTEM SOFTWARE (SPSS), Windows version 10.0, 1999, New York. SPSS Inc.

Results

The females of *L. punctata* start laying eggs in clutches by the end of August and continued until October. The regressive period corresponded with winter (September–February); both the sexes exhibited low physical activity and underwent hibernation, which continued until the onset of summer (March) (Table 1).

Observations on plasma glucose levels

During the regressive period, fasting plasma glucose level was the lowest (91.39 ± 2.76 mg%). It increased to 96.87 ± 3.63 mg% during the regenerative period and reached a peak value of $119.27 + 1.68$ mg% during the reproductive period (Fig. 1). There was no significant variation in the plasma glucose level between the sexes. However, such a variation existed when the data for mean glucose level were compared between regenerative and reproductive, and reproductive and regressive periods. An *F*-value of 34.22 with 2 and 85 degrees of freedom was found to be highly significant ($P < 0.01$). Between the regressive and regenerative periods the differences were not significant.

Light microscopic observations

The pancreas showed a number of islets of varying sizes in all its regions. Larger islets were seen in the splenic region with dense endocrine cells and more blood capillaries containing red blood cells besides nerve innervations irrespective of the sex and season. The islets were irregular in shape and devoid of connective tissue capsules. They orientated towards the
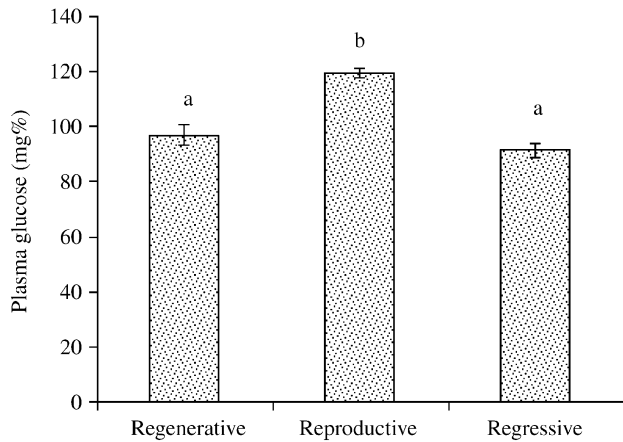
Fig. 1—Seasonal variation in plasma glucose in *Lissemys punctata*. Data are the means ± SD of 40 observations in each period. *P* < 0.01 between regenerative and reproductive; reproductive and regressive periods.

blood vessel or towards the ducts. Endocrine cells stained darker than the surrounding exocrine tissue under CHP staining. The length and weight of the pancreas also varied with season, being highest in the regressive period (Table 2). On average, the pancreas was 8.97 ± 1.9 cm in length and 1.539 ± 0.45 g in weight.

During the regenerative period, the endocrine cells were present throughout the pancreas as individual cells or as smaller aggregations between the acini. The islets had a mean size of 1.715 ± 1.1 mm. The total β-cell volume was 71.67 ± 10.28% of the islets. The pancreas of the reproductive animals also had endocrine cells throughout but in smaller clumps between the exocrine tissues. The mean size of the islet measured 0.83 ± 0.3 mm. The volume of β cells was 67.99 ± 17.8% in comparison to the proportion during the regenerative period. During the regressive period, the pancreas of *L. punctata* showed a higher number of larger islets throughout, with tightly packed endocrine cells. The average size of the islet was 2.659 ± 1.8 mm and the volume of β cells

was 84.91 ± 8.8% of the islet. During this period, the islets were also found concentrated towards pancreatic periphery. No individual endocrine cells were seen between the exocrine tissues. Macrophages were a common occurrence in the pancreas of these animals.

β cells were ovoid, blue-stained, with central oval or lobed nuclei, rich in haematoxylin-positive granules. The cells were, on average, 4.7 ± 0.95 μm at their longest axis. The islet cells were smaller or equal to the size of nuclei of exocrine cells. The β-cell size remained the same irrespective of the pancreatic region, sex and age of the animals, but some of the largest, measuring up to 7 μm, were found in regressive periods. The size of the islets varied from 0.2 mm to 9.8 mm with a mean value of 2.52 ± 1.8 mm at their longest axis. Morphometric analysis showed that the β cells were the most abundant, amounting to approximately 73% of the endocrine pancreatic mass.

Analysis of data indicated that there was a significant difference in mean β-cell volume and cell count between the periods of the annual reproductive cycle. The averaged values represent orders of magnitude rather than fixed levels, as the range of variability was great between individuals of this population.

Discussion

The weight of the animal and its pancreas varied with season (Table 2). The structure of the pancreas of *L. punctata* remained the same as described by Miller and Lagios (1970). The colour of the pancreas varied from pinkish to yellowish depending on the phases of the reproductive cycle. The plasma glucose level in *L. punctata* starved for 2 months was 46 mg% (Dessauer 1970) whereas Padgaonkar and Mehendale (1986) reported 79.98 mg% after 48-h starvation in *L. punctata granosa*. In the present investigation, higher values are exhibited (91.39–119.27 mg%) for this parameter during different periods of reproduction, although the period of starvation was comparable with that used by Padgaonkar and Mehendale (1986). The difference in the plasma glucose

Table 2 Morphometric measurements of animal and pancreas in different periods of the reproductive cycle

Parameter	Reproductive periods			Statistical analysis	
	Regenerative	Reproductive	Regressive	*F*-value	*P*-value
Animal weight (g) (mean ± SD)	1460 ± 432.56	1560 ± 424.78	1390 ± 284.60	0.488	0.619
Carapace length (cm) (mean ± SD)	22.6[a] ± 2.1	24.7[b] ± 1.9	21.9[a] ± 1.7	5.218	0.012
Pancreas weight (g) (mean ± SD)	1.509 ± 0.64	1.532 ± 0.34	1.577 ± 0.32	0.067	0.936
Pancreas length (cm) (mean ± SD)	8.57 ± 1.74	8.44 ± 1.8	9.91 ± 1.9	1.729	0.196

Means with same letters are not significantly different from each other.

level recorded by these investigators and that observed by the present investigators could be attributed chiefly to differences in the diet fed to the experimental animals, to different periods of reproduction and to their ecotypes. Obviously, a highly reduced plasma glucose level recorded by Dessauer (1970) is an impact of prolonged starvation possibly coupled with differences in the diet as well as the ecotype of *L. punctata*. Under the CHP method, the islets were darkly stained as compared to mammalian islets. They were irregular, without connective tissue capsules. Comparable findings were also recorded in the turtles *Testudo graeca* L. and *Mauremys caspica* (Garcia Ayala *et al.* 1987, 1989). Endocrine cells in *L. punctata* were smaller or similar to the size of acinar cell nucleus. In the islets, β cells usually occupy the central region surrounded by other cell types. Most of the islets were orientated towards the blood capillaries, indicating their endocrine property. The islet cells in *L. punctata* were present throughout the pancreas either as individual cells, as small groups, or as large clusters, splenic islets being the largest. Gapp *et al.* (1985) found pancreatic endocrine cells in the turtle *Chrysemys picta* scattered as single cells or as small aggregates throughout the exocrine tissue. In turtles, crocodilians and certain squamata, the islets were scattered throughout the gland (Epple and Brinn 1987). Some differences in the distribution and morphological features of pancreatic endocrine cells have been observed in *M. caspica* during summer and winter despite not showing any decrease in vital activities (Garcia Ayala *et al.* 1989).

During the regenerative period of *L. punctata*, many β cells were seen individually and a few were in small clusters. Individual cells were less common in the reproductive period; they were not prominent in the regressive period. Interestingly, whenever the total β-cell count declined, the animals exhibited higher plasma glucose levels irrespective of the season. The females showed comparatively higher plasma glucose values and denser islet cell masses than the males in all seasons. The islets reported in the turtles *Pseudemys scripta* (Agulleiro *et al.* 1985), *Testudo graeca* L. (Garcia Ayala *et al.* 1987), and *Mauremys caspica* (Garcia Ayala *et al.* 1989) were mostly small but showed trends of developing into larger islets. Hoet *et al.* (1995) have reported that new β cells could be produced at the rate of 4–10% at different glucose concentrations in glucose-treated animals.

In *L. punctata*, the islets were also orientated towards the duct, particularly in the regressive period. Some of the larger duct walls exhibited the presence of β cells in them as a blue-stained cell mass. In cyclostomes, as reported by Epple and Brinn (1987), the islets develop directly from the bile duct cells which, according to Bonner-Weir (2000) could be facultative cells.

The average weight of the animals during the regressive period was low but the average weight of the pancreas remained comparatively high (Table 2). The size of the islets was also largest, with the highest number of endocrine cells. There was a negative correlation between islet mass and
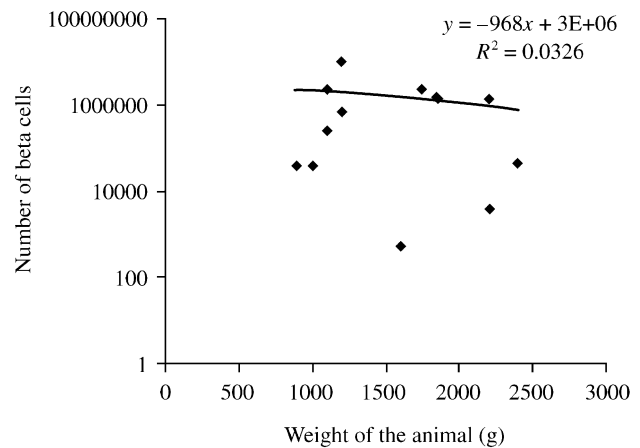

Fig. 2—Relationship between body weight and islet mass calculated by taking β-cell count as an indirect measure of islet mass in *Lissemys punctata*. Actual data of all animals used in the experiment irrespective of sex and season are given.

animal weight (Fig. 2) though it varied at the individual level. In general, the larger the animal, the smaller the islet size and cell count. β cells were found throughout the pancreas, being more numerous in the splenic region in *L. punctata*. These observations are in conformity with those reported in *M. caspica* in which insulin-secreting cells were more abundant in the splenic pancreas than in the duodenal region during winter, but were found distributed uniformly throughout the gland in summer (Garcia Ayala *et al.* 1989). By contrast, the distribution of these cells did not differ between summer and winter in *T. graeca* (Garcia Ayala *et al.* 1987). *Lissemys punctata* during the regressive period showed the lowest plasma glucose, higher glycogen localization in the liver and excessive white and brown fat deposition in the abdominal cavity; the pancreas appeared predominantly pinkish because of its higher blood supply and highest β-cell count. Fat accumulated during the regressive period underwent a steady decline from the regenerative to the reproductive period, indicating its utilization in the reproductive activity of the animals. The basal metabolic rate in poikilotherms during hibernation is maintained by higher levels of thyroxin from the thyroid gland (Lynn 1970). Thyroxin promotes the breakdown of glucose and brown fat to provide energy, which is important for hibernating animals because it helps to raise the core temperature rapidly during waking from hibernation (Taylor *et al.* 1997). The thyroid gland of regressive animals of *L. punctata* also exhibited higher colloidal content in the follicles.

In the present investigation, some of the β cells of *L. punctata* during the regressive period pooled their granules into the blood capillaries and were seen as accumulations of secretory granules on the exterior of the cell membrane. Most of the β cells grew larger during this period; some of these were stained pale blue, presumably because they had emptied their secretory content. Hypertrophy of the cells was to meet

increased demand for insulin to enhance glucose uptake by peripheral tissues. There was a marked increase in the number of macrophages in the vicinity of the islets, probably to weed out the older β cells. Hypertrophy of the cell is reported to be a sustained amplification of gene expression without cell division (Bonner-Weir 2000).

The β cells were less abundant during the regenerative period and became least abundant during the reproductive period. Plasma glucose level was higher during the reproductive period as opposed to the regenerative and regressive periods. A rise in temperature is reported to increase glucose level in alligators and in the snake *Vipera aspis* (Dessauer 1970) as well as in *L. punctata* (Ray and Maiti 2001), but to cause a fall in glucose in the turtle *Emys orbicularis* (Dessauer 1970); glucose rises sharply in *Pseudemys* subjected to a sudden drop in temperature (Dessauer 1970). During the transition from the reproductive to regressive period, blood glucose might have influenced the proliferation of β cells in *L. punctata*. Higher blood glucose levels in this turtle may be attributed to larger numbers of insulin-producing β cells, which were in a hypertrophied condition. Insulin secretion from these cells may have a significant role to play in the carbohydrate metabolism in *L. punctata*. The β-cell population has a slow turnover, with cell renewal coming from replication of pre-existing β cells and from differentiation of cells that act as precursors in the adult ductal epithelium (Bonner-Weir 2000). In the present study, it was observed that β-cell mass remained dynamic. Though β-cell mass along with non-β cells increased significantly from the reproductive to the regressive period, there was no evidence for mitotic cell division. There is experimental evidence that β-cell mass can increase or decrease (Bonner-Weir 2000) and they must have a finite life span like other cell types. Meanwhile, regenerative and early reproductive animals had little or no fat deposition in and around the viscera. The pancreas appeared yellowish, making it difficult to separate the organ from duodenum whenever the animals had increased fat deposition in and around the viscera. The animals became voracious eaters after the termination of hibernation and only excess carbohydrate was deposited as fat. With the increase in ambient temperature from March onwards they became more active and initiated gonadal activity, which reached its peak in the reproductive period. Owing to the lesser density of β cells, the plasma glucose increased progressively from the regenerative to the reproductive period. Variation in the colour of the pancreas depended on blood supply, which in turn appeared to be dependent on endocrine activity, which was higher during the regressive period when *L. punctata* had significantly higher plasma glucose (average 102.51 ± 5.37 mg%). Unsurprisingly, Dessauer (1970) has reported a highly reduced (33–91 mg%) plasma glucose level when the testudians were subjected to starvation for a period of 2 months. The plasma glucose estimated for the reptile *Chrysemys dorbigini* during summer and winter ranged from 76 to 95 mg%. He also observed that plasma insulin reached its peak in winter when

blood glucose was at a minimum. Similarly, a comparable plasma glucose level (69.97–119.27 mg%) was observed in this reptile (*C. dorbigini*) during different periods of reproduction (Correa *et al*. 1960).

The organization of blood sugar levels in reptiles is poorly understood. Along with other metabolic demands, glucose is also a stimulant factor for β-cell regeneration and vice versa. Change in plasma glucose is dependent on β-cell number and physiological events of the reproductive cycle. This in turn depends on the ambient temperature and/or seasonal cycle of the animal in a particular location.

Acknowledgements

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