Histochemical analysis of the thecal interstitial gland in the ovaries of the microchiropteran bat, *Hipposideros speoris* (Schneider) during the reproductive cycle

M.S. Sastry, S.B. Pillai and D. Tembhare
Department of Zoology, RTM Nagpur University, Nagpur-44003, India.

Summary

A study of ovaries during a complete reproductive cycle in mature females of *Hipposideros speoris* showed the presence of thecal type interstitial gland tissue during the late-recrudescence and breeding phases. Luteal-like transformation of fibroblasts of theca of multilaminar atretic follicles results in the formation of thecal interstitial gland tissue. This interstitial tissue undergoes a variety of cellular alterations till its complete differentiation. After persisting for a short while in the wall of degenerating follicles it reverts back as stromal cells and thus is very transient. Cyclical variations in the histochemical localization of lipids, 3β-hydroxysteroid dehydrogenase (3β-HSDH), 17β-hydroxysteroid dehydrogenase (17β-HSDH), succinic dehydrogenase (SDH) and Glucose-6-phosphate dehydrogenase (G-6-PDH) were observed. The histochemical shift in the enzyme activities and substrates during the reproductive cycle, corresponding to changes in the metabolic requirements as well as steroidogenesis suggests role for this tissue in ovarian physiology.

Key words: Microchiroptera, *Hipposideros*, ovary, thecal interstitial gland

Introduction

Of the four major steroidogenic components of mammalian ovary, the interstitial gland tissue is the most controversial and least studied. Three types of interstitial gland tissue were observed in the ovaries of the microchrpoteran bat *Hipposideros speoris*, the thecal type, stromal type and epithelial cords, all of which exhibit cyclical variations histologically and histochemically in relation with reproductive cycle (Sastry and Pillai, 2005; 2008). The thecal type of interstitial tissue is formed by the hypertrophy of the theca and the surrounding stroma of degenerating multilaminar follicles. Most of the mammalian species studied so far showed this type of interstitial tissue in their ovaries (Mossman and Duke, 1973; Bernard and Meester, 1982; Rasweiler, 1988; Trivedi and Lall, 2004; 2005; Singh et al., 2005). The steroidal secretions of these interstitial tissues may play an important role in the initiation of sexual maturation and subsequent regulation of cyclical activity of ovary, about which little is known. The present study provides a brief account of thecal interstitial gland tissue and enzymatic activity which have not been described by any other author in details excepting Singh et al. (2005).

Material and Methods

Collections of animals and histological study

The study was approved by the Departmental Research Committee, RTM University, Nagpur, India. More than 20 specimens of *Hipposideros speoris* were collected once every month for one year from the natural population inhabiting the abandoned mines in Khapa (20°92"N, 78°95"E), Nagpur, Maharashtra. This bat is found to inhabit in cold and humid places, preferably dark. All the bats from the same colony do not show same pattern of reproductive behavior at any given time because there exists an asynchrony in reproductive cycle among different females of the same colony. For histological studies the ovaries were fixed quickly in Bouin's fixative, dehydrated in ethanol and embedded in paraffin wax. Sections were cut at 5mm thickness and stained with hematoxylin and eosin.

Correspondence to be addressed to : Dr. M.S. Sastry, Ph.D., Email: sastry_nidhi@rediffmail.com

Histochemical detection of lipids

Ovaries fixed in formol–calcium were sectioned (10mm) in a freezing microtome and stained adopting Chiffle and Putt method (Lillie and Fullmer, 1976). The sections were washed briefly in water and stained with Sudan black B for general lipids. Lipids appeared black or bluish black. However, in the oil red O technique, Sudan black B was substituted with oil red O, when lipids appeared red.

Histochemical detection of enzymes

For histochemical localization of hydroxysteroid dehydrogenases (3β, 17β); glucose-6-phosphate dehydrogenase (G-6-PDH) and succinic dehydrogenase (SDH), sucrose-fixed tissues were cut in cryostat (-20°C) at 10mm thicknesses. The incubation medium for 3β-HSD consisted of nitroblue tetrazolium (nitro BT), Nicotinamide adenine dinucleotide (NAD^+) and pregnenolone dissolved in 2-2 dimethylformamide, for 17β-HSD it consisted of testosterone dissolved in 2-2 dimethylformamide. For G-6-PDH the substrate was glucose-6-phosphate dissolved in 2-2 dimethylformamide, and for SDH it was di-sodium succinate. The reaction product was visualized by conversion of nitro-blue tetrazolium to tetrazolium granules. Appropriate controls were run in substrate-deficient media. The color intensity of the reaction product was scored as +++ (strong), ++ (moderate), + (low), – (negligible) and – – (no reaction) (Table-1).

Observations

During recrudescence and breeding phases of the reproductive cycle a large number of atretic multilaminar and antral follicles were observed. Along with the degenerative changes in the oocyte and granulosa, the thecal cells of most of the larger follicles underwent degeneration, but in few multilaminar and vesicular follicles they remained intact and underwent progressive changes, ultimately forming the interstitial gland cells. The hypertrophied theca of these antral and multilaminar atretic follicles resulted in a thick band of cells around the atretic granulosa cells (Figs. 1, 2).

The fibroblast elements of the thecae along with adjacent stromal cells of most of the atretic vesicular and some of the secondary follicles underwent luteal-like transformation to form the interstitial gland tissue. These glands were mostly encountered during late-recrudescence (October and early November) and breeding phase (late November and December). A slight enlargement in a few of the thecal cells characterized the earliest phase of the interstitial gland development. The cells were characterized by granular cytoplasm and small compact nuclei (Fig. 3). The interstitial cells soon became less compact, and the dispersed nuclei came close and showed vesiculation. The cytoplasm got localized around the nuclei. The cell membrane also differentiated and, thus, formed syncytial or multicellular arrangements (Fig. 4). Further more cellular differentiation resulted in a distinct zone of interstitial tissue. These tissues consisted of large polygonal compactly arranged cells, forming 3-4 concentric layers (Fig. 5).

This type of interstitial gland cells were very transient in nature, and by the end of the breeding phase, i.e., during mid-December, the cells degenerated and reverted to stromal tissue. The degeneration was characterized by the diminution of the cell size and pyknosis of the nuclei. This left small elongated cells and increased inter-cellular spaces between the cells (Fig. 6). The thecal interstitial gland tissue was thus very short-lived and, hence, its degeneration could not be authenticated. Being transitory in nature the progressive development and regression of these cells were not traceable.

The differentiation of the thecal type interstitial gland tissue was associated with high vascularity and accumulation of large amounts of sudanophilic lipid droplets in the hypertrophied thecal cells and surrounding blood vessels (Fig. 7) during recrudescence (September-October) whereas the activity appeared diffuse (Fig. 8) with the approach of breeding period (November). The reaction profile of 3-β-HSDH was in consonance with the lipid staining, low during recrudescence (Fig. 9) but well characterized by the appearance of formazan granules in the fully differentiated thecal cells (Fig. 10). The reactivity of the other steroidogenic enzyme, 17-β-HSDH, was in similar as for 3β-HSDH during the described stages (Figs. 11, 12). The mitochondrial enzyme succinic

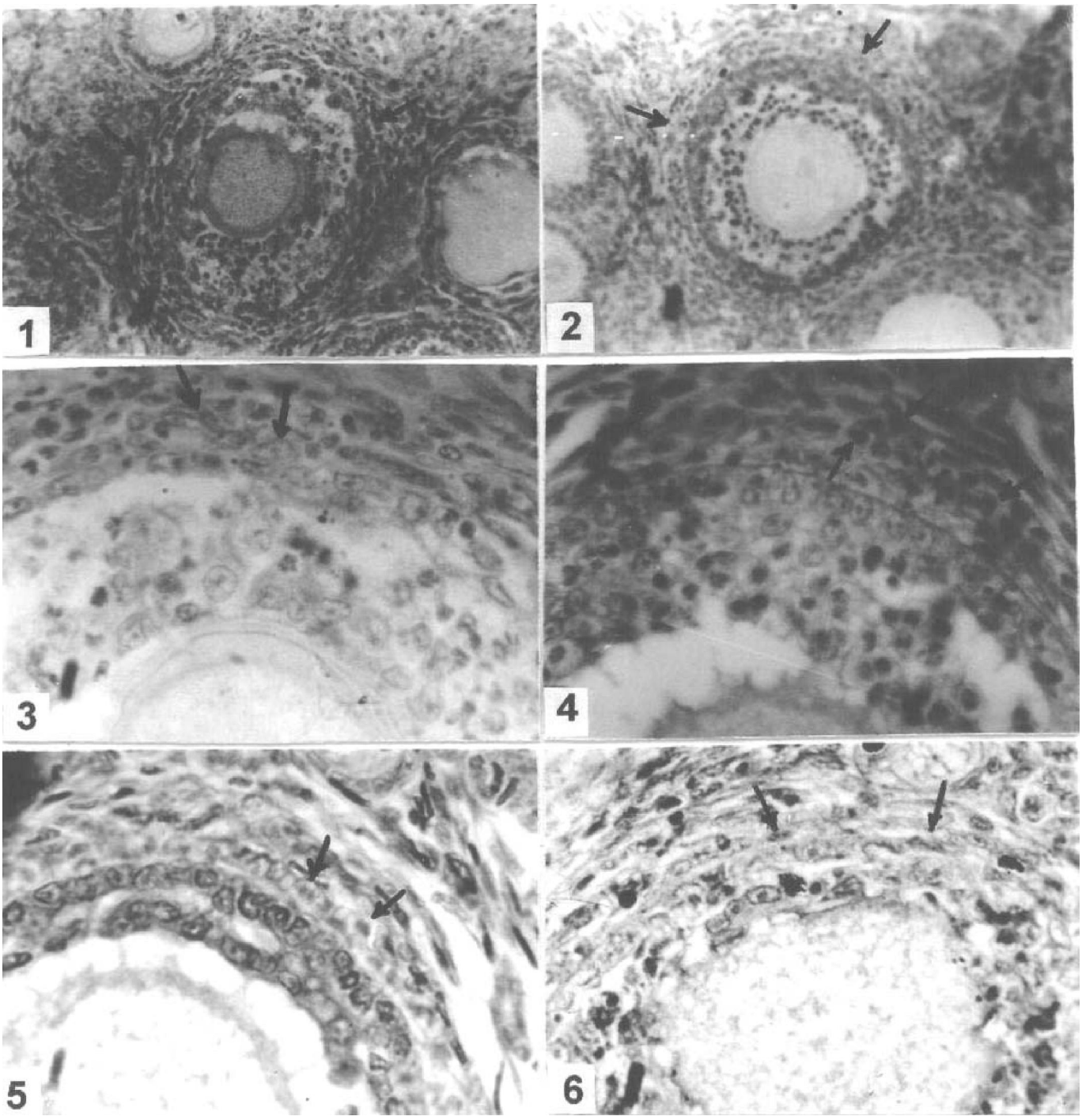

Fig. 1. Large antral follicle in the tertiary stage of atresia in an early recrudescent ovary illustrating initial step in the interstitial tissue formation (arrow). x100.

Fig. 2. Note a thick band of interstitial tissue (arrow) around the atretic secondary follicle (early November). x100.

Fig. 3: Thecal interstitial tissue (arrow), each cell with granular cytoplasm and small nuclei X250.

Fig. 4: Thecal interstitial tissue with well developed cells showing distinct, rounded nuclei (arrow). x250.

Fig. 5: The hypertrophied polygonal interstitial cells arranged closely showing large amount of cytoplasm and no distinct cell border (arrow) during breeding phase (November). x250.

Fig. 6: Thecal interstitial gland cells showing regression (early December). Note the cells diminished in size, with pyknotic nuclei and large intercellular spaces. x250.

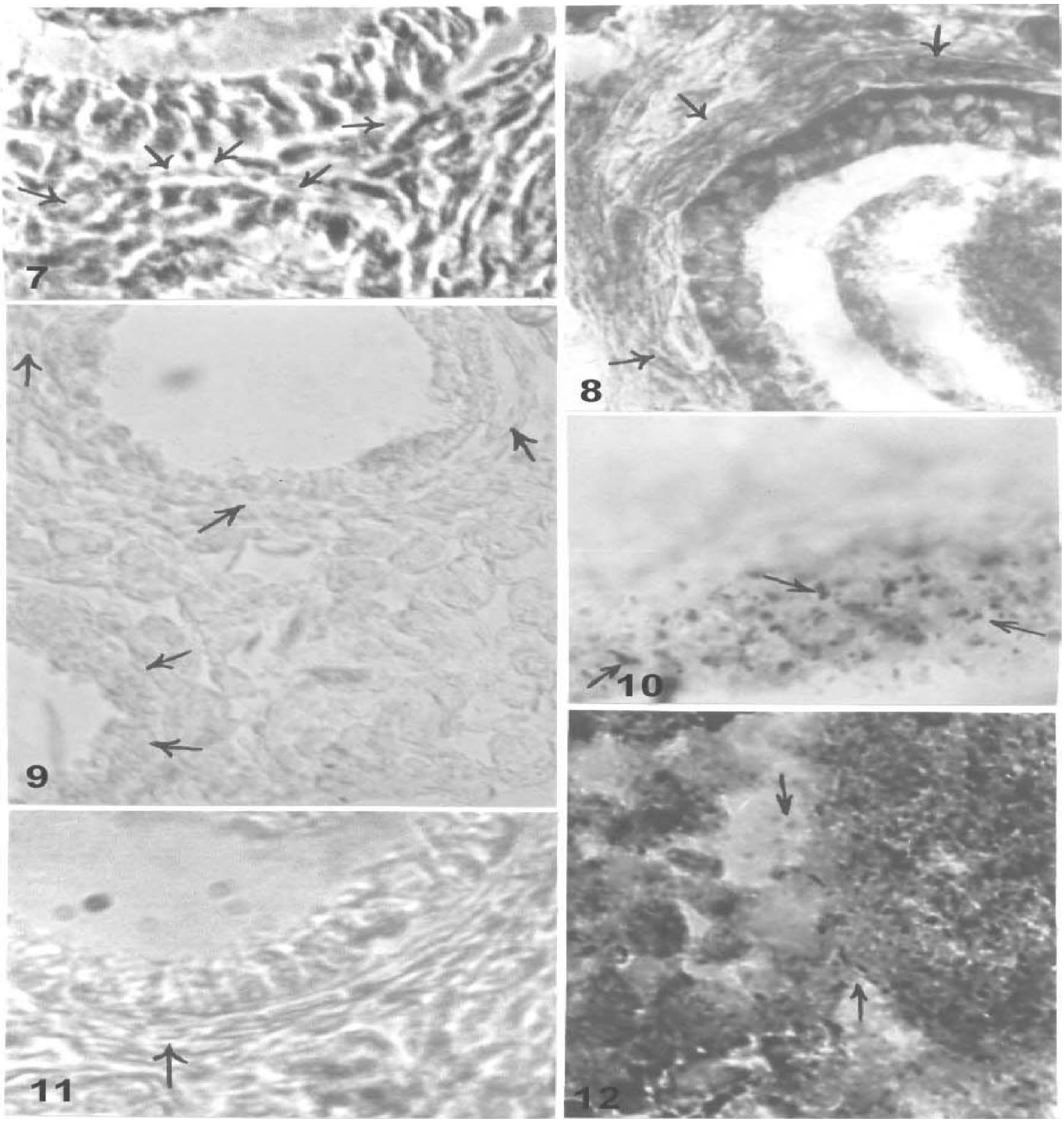

Fig. 7: The developing theca having negligible deposition of lipid around the early multilaminar atretic follicle (arrow). Atresia is evident by the disrupted stratum granulosum (early recrudescence, September). x1000.
Fig. 8: A thick band of thecal interstitial gland tissue exhibiting diffuse sudanophilic lipid droplets (arrow) during November. Note large accumulation of lipid droplets in the blood vessel (arrowhead). x250.
Fig. 9: Formazan granules deposition (arrow) in the thecal luteal gland cells (late recrudescent phase, October). x450.
Fig. 10: Wall of an atretic tertiary follicle showing strong formazan granules in the cytoplasm of the thecal interstitial cells during breeding phase (arrow). x250.
Fig. 11: Fully developed thecal glands during late-recrudescence, showing low to moderate reactivity (arrow) for 17β-HSDH. x1000.
Fig. 12: Thecal interstitial glands of pre-ovulatory phase (arrow). Note marked intensity of 17β-HSDH as demonstrated by strong formazan granules (arrow). x400.

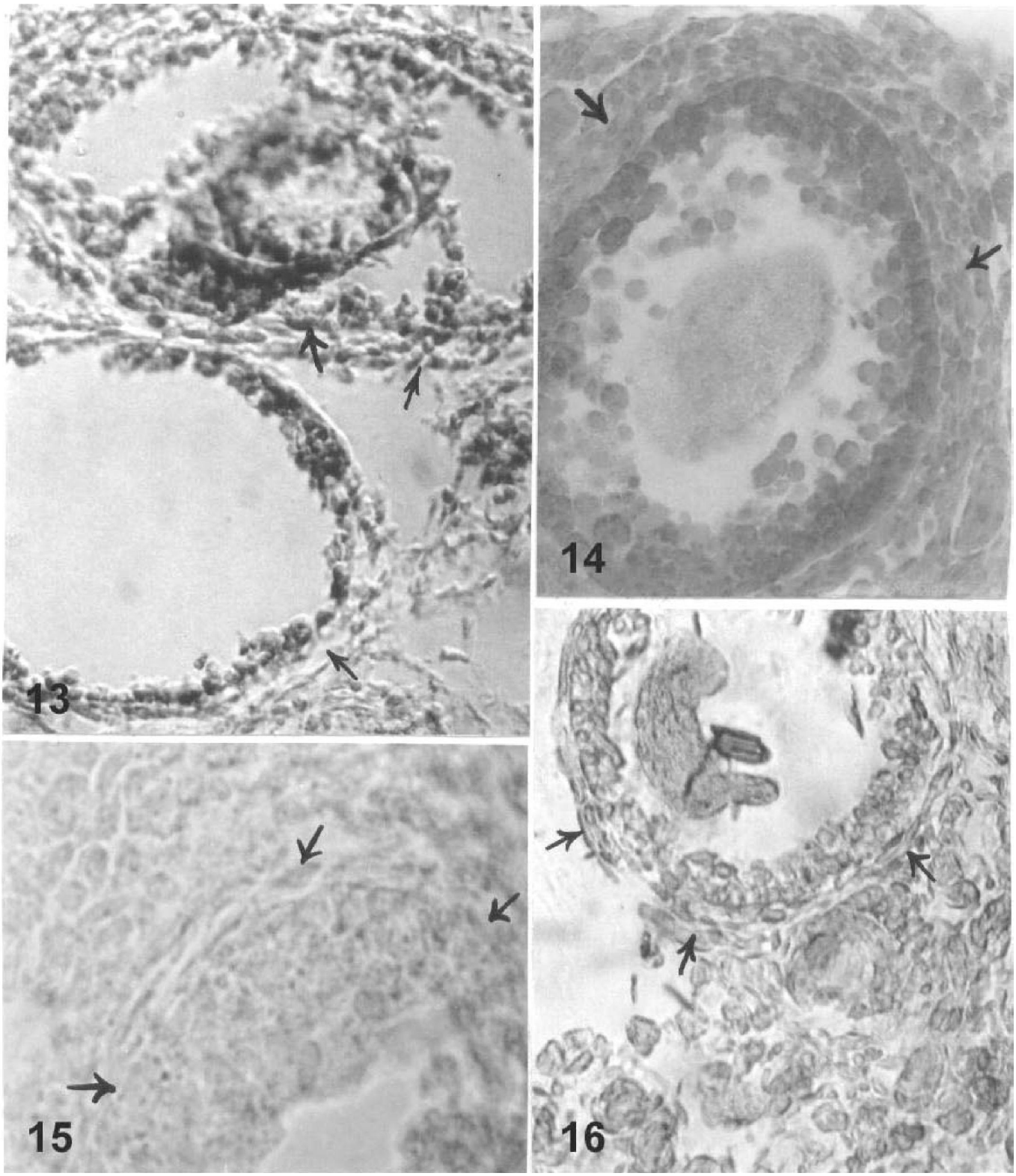

Fig. 13: Slight SDH activity is detectable in thecal interstitial gland during recrudescent stage of breeding cycle (arrow). x400.
Fig. 14: Thecal interstitial gland tissue illustrating high succinate dehydrogenase activity (arrow) during breeding phase (November). x400.
Fig. 15: Recrudescent ovary (early October) stained for G-6-PD demonstrating an enhancement in the development of thecal interstitial gland cells and staining profile for the enzyme (arrow). x1000.
Fig. 16: The increasing activity of G-6-PD in thecal gland from a specimen collected during mid-November (arrow). x450.

Table 1. The histochemical characterization of thecal interstitial gland cells in relation to its development during recrudescence and breeding phases of reproductive cycle in the ovary.

Chemical substance studied in thecal gland	Phases of reproductive cycle	
	Recrudescent	Breeding
Diffuse lipo-protein (Sudan black B)	+	++
3β-HSDH	++	++++
17β-HSDH	++	++++
SDH	++	+++
G-6-PDH	++	+++

dehydrogenase (SDH) exhibited similar profile of intensity as described above during both the phases of the ovarian cycle (Figs. 13, 14). The profile of these enzymes was substantiated by the other mitochondrial enzyme G-6-PDH (Figs. 15, 16).

Discussion

The appearance of thecal interstitial gland tissue only during recrudescence and breeding phases observed in this study has been earlier described in other bats, *Hipposideros caffer* (Mossman and Duke, 1973; Bernard and Meester, 1982), *Otomops martiensseni* (Kayanja and Mutere, 1975), *Scotophilus heathi* (Singh and Krishna, 1994; Singh et al., 2005), *Antrozous pallidus* (Oxberry, 1977), *Noctilio albiventris* (Rasweiler, 1979), *Glossophaga soricina* (Rasweiler, 1972), *Rhinolophus capensis* (Bernard, 1985), *Molossus fortis* (Krutzsch and Crichton, 1985), *Rinolaphus eurale* (Tsvetkov and Takeva, 1988), *Molossus ater* (Rasweiler, 1988) and *Rhinopoma microphyllum kinneari* (Trivedi and Lall, 2004, 2007). However, these earlier reports did not specify the reproductive status of the ovary, and origin, development and functional status of the thecal interstitial tissue, excepting Singh et al. (2005).

The thecal type interstitial cells originate due to hypertrophy or transformation of theca interna of atretic vesicular and multilaminar follicles in *Hipposideros speoris*. The thecal interstitial glands are of very transient nature in *Hipposideros speoris* as in a few other cases such as woman, rhesus monkey, cow and buffalo, as they quickly revert to embryonic stromal tissue and appear to be regulated by various factors such as the nature and amount of gonadotropins, blood and nerve supply, genetic and metabolic factors, etc., (Guraya, 1985, 2000). Besides reverting back to the original stromal tissue, some interstitial tissue in *Hipposideros speoris* was also observed to undergo degeneration. The presence of varying amounts of diffuse lipids and lipid droplets in the interstitial tissue signifies its importance as steroidogenic tissue since lipids serve as the potential precursor substance for steroid biosynthesis. The interstitial tissue of *Hipposideros speoris* reacted positively for the steroidogenic enzymes 3β-HSDH, 17β-HSDH, SDH and G-6-PDH during recrudescence and breeding. Such enzyme activities are indicative of steroid synthesis as has been demonstrated in the interstitial gland cells of thecal origin of some bats (Singh and Krishna, 1994; Singh et al., 2005; Trivedi and Lall, 2004, 2007).

The positive reactivity of the steroidogenic enzymes during recrudescence and breeding phases, in this study, corroborates with the reports of Mc Natty et al. (1984), Erickson et al. (1985), Bergh et al. (1993) and Singh et al. (2005), who described production of androgen, which is essential for the maintenance of the structural integrity of ovary, its cyclical activity via recruitment of primary follicles, and selection of dominant follicle by atresia, since *Hipposideros speoris* is a mono-ovulator.

Thus, it is concluded that the process of atresia in the antral and secondary follicles in *Hipposideros speoris* is related to the formation of thecal type interstitial gland cells, which in turn maintain the ovarian integrity.

References

Bergh C, Olsson JH, Slleskog U, Hillensjo T (1993) Steroid production in cultured thecal cells obtained from human ovarian follicles. *Hum Reprod*. **8**: 519-524.

Bernard RTF (1985) Reproduction in the cape horse-shoe bat (*Rhinolophus capensis*) from South Africa. *S Afr J Zool*. **20**: 129-135.

Bernard RTF, Meester JAJ (1982) Female reproduction and the female reproductive cycle of *Hipposideros caffer caffer* (Sundevali, 1846) in Natal, South Africa. *Ann Trans Mus Ann Van die Transvaal- Museum*. **33** : 131-144.

Erickson GF, Magofin DA, Dyer CA, Hofeditz C (1985) The ovarian androgen producing cells: a review of structure relationships. *Endocr Rev*. **6:** 371-399.

Guraya SS (1985) *Biology of Ovarian Follicles in Mammals*. Berlin, Heidelberg, New York**:** Springer.

Guraya SS (2000) *Comparative Cellular and Molecular Biology of Ovary in Mammals: Fundamental and Applied Aspects*. Pp 195-236. India: Oxford and IBM Publishing Co., Pvt. Ltd.

Kayanja FIB, Mutere F (1975) The ovary of the insectivorous bat, *Otomops martiensseni*. *Anat Anz*. **137**: 166-175.

Krutzsch PH, Crichton EG (1985) Observations on the reproductive cycle of female *Molossus fortis* (Chiroptera: Molossidae) in Puerto Rico. *J Zool*, London (A). **207**: 137-150.

Lillie RD, Fullmer HM (1976) *Histopathologic Technic and Practical Histochemistry*. 4th Edition. New York: McGraw-Hill Book Company.

McNatty KP, Health DA, Lun S, Fannin JM, McDiarmid JM, Henderson KM (1984) Steroidogenesis by bovine theca interna in an in vitro perfusion system. *Biol Reprod*. **30**: 159-170.

Mossman HW, Duke KL (1973) *Comparative Morphology of the Mammalian Ovary*. Madison: Univ Wisconsin Press.

Oxberry BA (1977) Ovarian morphology and steroidogenesis in the pallid bat, *Antrozous pallidus*. *Anat Rec*. **187**: 673 (Abs).

Rasweiler JJ (1972) Reproduction in the long-tongued bat *Glossophaga soricina*. *J Reprod Fertil*. **31**: 249-262.

Rasweiler JJ (1979) Bats as models in studies on folliculogenesis, menstruation, early pregnancy and sperm survival. In: Alexander N (Ed) Proceedings of Symposium on Animal Models for Research on Contraception and Fertility. Pp 437-446. Hagerstown, Maryland: Harper and Row Publishers.

Rasweiler JJ (1988) Ovarian function in the captive black mastiff bat, *Molossus ater*. *J Reprod Fertil*. **82**: 97-111.

Sastry MS, Pillai SB (2005) Cyclical changes in the epithelial cords of ovary of a microchiropteran bat, *Hipposideros speoris* (Schneider). *J Endocrinol Reprod*. **9**: 37-42.

Sastry MS, Pillai SB (2008) Variations in the epithelial cords of ovaries of microchiropteran bat, *Hipposideros speoris* (Schneider) during reproductive cycle: an enzymatic approach. *J Theor Exp*. **4**: 95-101.

Singh UP, Krishna A (1994) Seasonal changes in the cytochemical and ultrastructural features of the ovarian interstitial tissue in a vespertilionid bat, *Scotophilus heathi*. *J Reprod Biol Comp Endocrinol*. **6:** 33-46.

Singh UP, Krishna A, Smith TD, Bhatnagar KP (2005) Histochemical localization of enzymes and lipids in the ovary of a vespertilionid bat *Scotophilus heathi* during the reproductive cycle. *Braz J Zool*. **65**: 179-186**.**

Trivedi S, Lall SB (2004) Ovarian dehydrogenases of the non-pregnant, pregnant and lactating *Rhinopoma microphyllum kinneari* (Chiroptera: Rhinopomatidae). *Vespertilio*. **8:** 105-112.

Trivedi S, Lall SB (2007) Histoarchitecture and λ -3β hydroxysteroid dehydrogenase profile in ovaries of the non-pregnant, pregnant and lactating insectivorous rat-tailed bat, *Rhinopoma microphyllum kinneari*. *Anim Biol*. **57:** 97-114.

Tsvetkov T, Takeva T (1988) Changes in interstitial gland in the ovary of the bat *Rinolaphus eurale* Blas: Arousal from hibernation. *Cryobiology*. **25**: 502-507.