

## Asynchrony of the reproductive organs of the male vespertilionid bat, *Scotophilus heathi*: role of gonadotrophins

Amitabh KRISHNA and Kavita SINGH

Krishna A. and Singh K. 1999. Asynchrony of the reproductive organs of the male vespertilionid bat, *Scotophilus heathi*: role of gonadotrophins. Acta Theriologica 44: 123–131.

Changes in the LH-cells of the anterior pituitary in relation to the reproductive cycle in the male *Scotophilus heathi* (Horsfield, 1831) are described. Immunocytochemical studies of the pituitary suggested two annual peaks of LH accumulation and release each associated with a period of active spermatogenesis and testosterone secretion. Further, in vitro study showed differences in testicular responsiveness to LH stimulation during November (a period of active spermatogenesis) and December (winter dormancy). Present study further showed that FSH is also capable of stimulating androgen secretion from the testis. Testicular FSH responsiveness varies from recrudescence to winter dormancy and this may be responsible for maintaining high circulating androstenedione production in *S. heathi* during this period.

Department of Zoology, Banaras Hindu University, Varanasi 221 005, India, e-mail: akrishna@banaras.ernet.in.

*Key words:* *Scotophilus heathi*, LH-cell, reproductive asynchrony, FSH, immunocytochemistry, in vitro, testis

### Introduction

Seasonally reproductive species generally exhibit elevated plasma testosterone concentrations during the breeding season(s), and there is a corresponding synchronous stimulation of both spermatogenesis and sex accessory gland functions. However, in most temperate-zone vespertilionid bats, activation of the sex accessory glands and the onset of breeding occur after the period of peak spermatogenesis and plasma testosterone levels (Gustafson 1979, 1987). It is suggested that this unique feature of asynchrony between primary and secondary reproductive processes in these bats has evolved as a result of temporal dissociation of the exocrine and endocrine activities of the testis. Singh and Krishna (1996) and Krishna and Singh (1997) have demonstrated such asynchrony between primary and secondary reproductive processes in a subtropical vespertilionid bat, *Scotophilus heathi* (Horsfield, 1891). The results of these studies showed an unusually high circulating concentration of androstenedione during recrudescence and winter dormancy. Testicular weight and serum testosterone concentrations showed two

peaks during recrudescence and breeding phases but declined significantly during winter dormancy. Unlike the temperate-zone vespertilionid bat showing reproductive asynchrony, a second peak of testosterone and spermatogenesis in *S. heathi* during late January coincided with the period of mating. The high circulating concentration of androstenedione during winter dormancy may be responsible for maintenance of sperm in the epididymis (Singh and Krishna 1995), however low testosterone during this period suppresses spermatogenesis. Thus, the high circulating concentration of androstenedione but low testosterone level during winter dormancy may be responsible for reproductive asynchrony in *S. heathi* (Krishna and Singh 1997). The purpose of this study was to investigate the role of gonadotrophs in regulation of the unique pattern of testicular androgen production in male *S. heathi* during the reproductive cycle. Two experimental approaches were employed: (1) evaluation of pituitary gonadotrophs cells using immunocytochemistry, (2) assessment of in vitro responsiveness of the gonad to gonadotrophs. These approaches were adopted because earlier attempt to measure serum LH and FSH concentrations in this species using human kit was unsuccessful.

### Material and methods

All bats were trapped alive in the Banaras Hindu University campus and adjacent areas. Body weight of each bat was recorded. Males were sacrificed by decapitation as soon as they arrived in the laboratory. Based on the reproductive cycle of *S. heathi* (Krishna and Singh 1997), males were classified into the following stages:

1. Quiescence (May–August): Testis does not show spermatogenesis, accessory sex glands regressed and sperm absent in epididymis.
2. Recrudescence (September–October): Testis showing spermatogenesis, accessory sex glands increased in size and weight but no sperm in epididymis.
3. First peak of spermatogenesis (November): Testis showing active spermatogenesis, accessory sex glands increased in size and weight but remained nonsecretory and sperm present in the epididymis
4. Winter dormancy (Mid-December to Mid-January): Testis not showing spermatogenesis, accessory sex glands regressed but sperm present in epididymis.
5. Breeding (February–March): Testis showing spermatogenesis, accessory sex glands active and sperm present in epididymis.

The bats were sacrificed and their testis and pituitary glands were excised out from the body. After fixing for 24 hr in Bouin's fluid they were preserved in 70% alcohol. The pituitary and testis were weighed after submergence in 70% alcohol. The tissues were then dehydrated in ethanol, cleared in xylol and embedded in paraffin wax. Serial sections of the pituitaries were cut at a thickness of 6  $\mu\text{m}$ .

### Immunostaining

Pituitary LH-gonadotroph cells were identified by using the peroxidase-antiperoxidase technique (Sternberger *et al.* 1970). All sections were deparaffinized in xylene, hydrated through a graded series of ethanol and equilibrated in 0.01 M PBS (pH 7.4). The deparaffinized sections were treated with 3%  $\text{H}_2\text{O}_2$  in 10% methanol for 5 min in order to inactivate endogenous peroxidase activity. Thereafter, the sections were washed twice for 5 min each in 0.05M Tris-buffered saline (TBS, pH 7.6) containing 0.25% Triton X-100 followed by 30 min incubation in 2% normal goat serum in TBS-Triton to eliminate nonspecific binding. After 2 rinses of 5 min each in TBS the sections were incubated with the primary antibody preparation (Anti-h $\beta$ LH, 1:1000 dilution in TBS, obtained from NIDDK, Lot No.



AFP-54372) in a moist chamber for either 4 hr at room temperature or overnight at 4°C. After washing twice in TBS for 10 min each, the sections were incubated with sheep antirabbit  $\gamma$ -globulin (IgG, Sigma, 1:100 dilution in TBS) for 30 min at room temperature ( $26 \pm 2^\circ\text{C}$ ). After 2 rinses of 5 min each in TBS the sections were incubated with the peroxidase-antiperoxidase complex (Sigma 1:50 dilution in TBS) for 30 min at room temperature. After rinsing in TBS, the immunoreactive cells were visualized by using 3,3' diaminobenzidine tetrahydrochloride (DAB) as substrate in the presence of  $\text{H}_2\text{O}_2$ . Thirty milligram of DAB (Sigma) were used in 40 ml of 0.05 M Tris buffer containing  $10 \mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$ . The sections were then dehydrated routinely. Control slides were performed as follows: (1) use of normal rabbit serum (1:1000 dilution) or TBS to replace the primary antiserum; (2) use of specific antisera, previously absorbed with hLH ( $5 \mu\text{g}/\text{ml}$  of 1:1000 diluted antiserum), and (3) use of TBS to replace sheep antirabbit IgG. Nonspecific immunocytochemical reactions were minimal in these control stainings.

### Morphometric analysis

The cell size of LH gonadotrophs was measured by an ocular micrometer on pituitary sections subjected to ICC. The dimensions of the stained cell type appearing largest in several sections were measured. At least 15 different cells were measured from at least 10 males from each group.

### *In vitro* studies

The aim of this experiment was to compare the effects of LH and FSH either alone or together on testicular androgen production during November (period of peak steroidogenesis and circulating testosterone level) and December (period of low testosterone). Testes collected from bats during November and December were placed in cold ( $4^\circ\text{C}$ ) media-199, and excess fat and connective tissues were removed. After washing with incubation medium, the testes (one in two halves per tube) were cultured for 2 hrs at  $37^\circ\text{C}$  in 1 ml of media-199 containing 0.1% BSA and LH ( $1 \mu\text{g}/\text{ml}$ ) or FSH ( $1 \mu\text{g}/\text{ml}$ ) either alone or together (both  $1 \mu\text{g}/\text{ml}$ ). Media were saved after incubation at  $-20^\circ\text{C}$  until assayed for testosterone and androstenedione.

### Steroid radioimmunoassay

Testosterone and androstenedione levels in the incubation media were determined by radioimmunoassay. Highly specific testosterone antiserum was purchased from ICN Biomedical Inc. (Lot No. R-15P). Antibody for androstenedione was obtained from Dr Resko (Portland, USA). Radioactive testosterone was purchased from ICN Biomedical and androstenedione from Amersham. Assays of testosterone and androstenedione were performed on  $20 \mu\text{l}$  unextracted samples of incubation media as described previously (Abhilasha and Krishna 1996). All the samples for a particular hormone were run in one assay. Inter- and intra- assay coefficients of variation were less than 9.8% for all assays.

### Statistical analysis

The data were analysed by one-way analysis of variance followed by Fisher PLSD and Schiff's Test. Wherever appropriate Student's *t*-test and correlation coefficient were also applied.

## Result

### Pituitary gland and LH gonadotrophs activity

The variations in the pituitary and testicular weights during the reproductive cycle are shown in Table 1. Multiple comparison test showed no significant ( $r = -0.36, p > 0.05$ ) correlation between pituitary and testicular weight cycles.

Table 1. Body, testes and pituitary weights of male *S. heathi* during different reproductive stages. The following differences are statistically significant by Fischer PLSD and Schiff's test ( $p < 0.05$ ): body weight - b, c vs a, d, e; testes weight - a vs b, d; c vs b, d; e vs b, c, d; b vs d; pituitary - e vs b, c, d. Values are mean  $\pm$  SE.

Reproductive stages	Body weight (g) (n = 6)	Testes weight (mg) (n = 6)	Pituitary weight (mg) (n = 3)
a. Quiescence	34.33 $\pm$ 1.74	59.39 $\pm$ 4.57	0.75 $\pm$ 0.13
b. Recrudescence	45.00 $\pm$ 1.09	193.41 $\pm$ 5.62	0.62 $\pm$ 0.08
c. Winter dormancy	40.83 $\pm$ 1.01	103.16 $\pm$ 3.22	0.58 $\pm$ 0.08
d. Breeding	32.83 $\pm$ 0.70	153.73 $\pm$ 2.21	0.55 $\pm$ 0.003
e. End of breeding	31.16 $\pm$ 0.48	88.03 $\pm$ 2.48	1.01 $\pm$ 0.18
ANOVA	< 0.001	< 0.001	< 0.01

The LH-cells are large, oval or round, sometimes irregularly shaped, usually occurring close to capillaries. The cytoplasm is finely granular with the granules dispersed uniformly throughout the cell. The nucleus is either eccentrically or centrally located. The LH cells are scattered singly or in small groups throughout in pars distalis.

The morphology, granulation pattern and size of the LH-cells showed marked variation during different reproductive phases (Table 2). The LH-cells assume a distinctly rounded morphology during recrudescence (October) and winter dormancy (December), while they appear much more irregular shaped during other phases of the cycle. The LH-cells were distributed throughout the pituitary during the quiescence phase. The cells during this stage were small with little cytoplasm and lightly stained with antisera (Fig. 1a). An increase in size and intensity of LH immunoreactive cells was observed during October which coincided with the beginning of active spermatogenesis and enhanced steroidogenesis (Fig. 1b). During the period of peak testicular activity in November LH-cells showed sign of degranulation (Fig. 1c). LH-cells remained granulated during winter dormancy and they were slightly larger in size and more intensely stained with antisera (Fig. 1d). During the period February–March again there was a decrease in the LH-cell size and immunoreactivity as compared to that in winter dormancy (Fig. 1e).

Table 2. Seasonal changes in the LH-cell size in the pituitary of male *S. heathi*. Difference between quiescence, peak testosterone, and breeding vs recrudescence and winter dormancy are statistically significant ( $p < 0.05$ ). Values are mean  $\pm$  SE.

LH - cell size ( $\mu\text{m}$ ) (n = 10)				
August (Quiescence)	October (Recrudescence)	November (Peak testosterone)	Early January (Winter dormancy)	Early March (Breeding)
11.04 $\pm$ 0.74	15.12 $\pm$ 0.85	11.75 $\pm$ 0.72	15.23 $\pm$ 0.79	9.87 $\pm$ 0.27



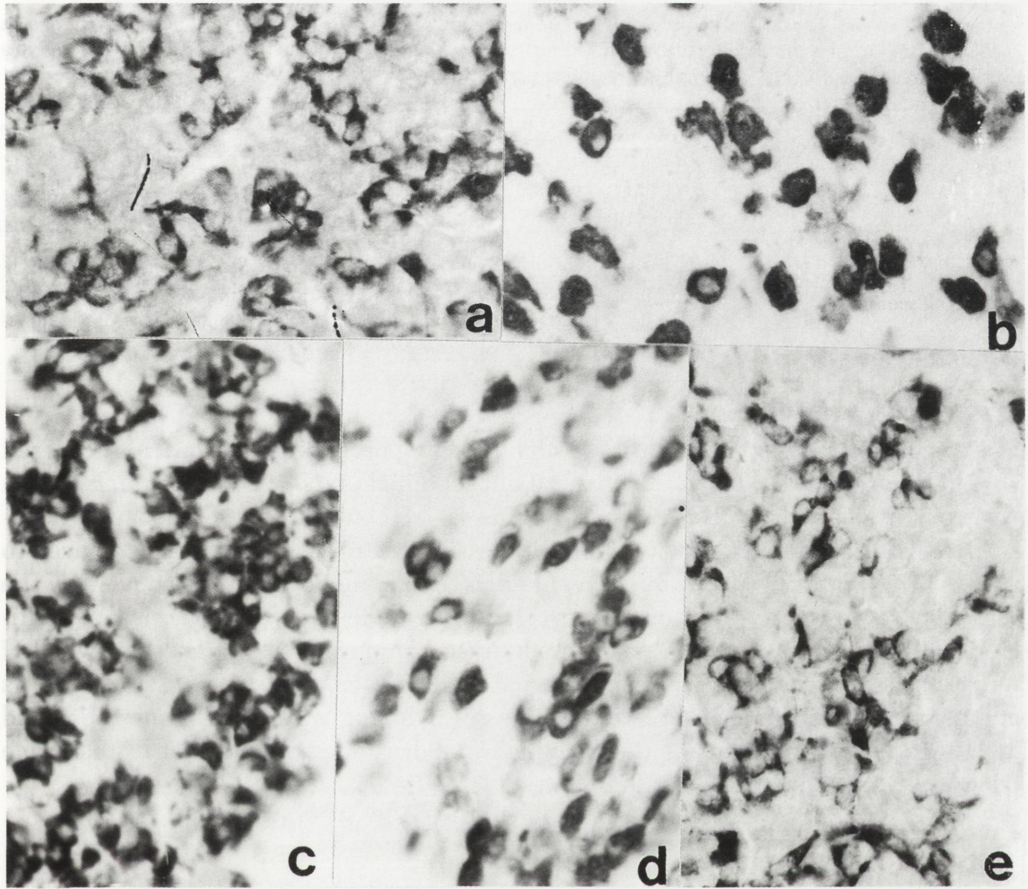


Fig. 1. The transverse sections of the anterior pituitaries of male *S. heathi* stained immunocytochemically with antisera against  $\beta$ LH.

a. Section of quiescence stage (August) showing small and lightly stained LH-cells containing little cytoplasm  $\times 180$ .

b. Section of recrudescence stage (October) showing increase in the intensity of immunostaining and the size of the LH-cells  $\times 180$ .

c. Section during period of peak testosterone synthesis (November) showing the degranulation of LH-cells and decline in the size of the cell  $\times 180$ .

d. Section of winter dormancy stage (December) showing the granulated and slightly larger size of LH-cells  $\times 180$ .

e. Section of breeding stage (March) showing the LH-cells. Note the decline in the size of the cell  $\times 180$ .

#### Effects of LH and FSH on testicular testosterone and androstenedione synthesis *in vitro*

This experiment was designed to compare the effects of LH and FSH on testicular testosterone and androstenedione production during the period of November (during peak serum testosterone and androstenedione concentration) and December (during the period of high androstenedione but low testosterone

Table 3. Effect of LH and FSH (1.0  $\mu\text{g/ml}$ ) on androstenedione and testosterone production *in vitro* in *S. heathi*. Differences between values marked by "a" vs control and values marked by "b" vs LH are statistically significant ( $p < 0.05$ ). Values are mean  $\pm$ SE.

Treatment	Sample size	Androstenedione (ng/ml)		Testosterone (ng/ml)	
		Recrudescence	Winter dormancy	Recrudescence	Winter dormancy
Control	4	6.15 $\pm$ 0.41	2.30 $\pm$ 0.15	6.98 $\pm$ 2.44	0.91 $\pm$ 0.06
LH	4	31.08 $\pm$ 5.96 <sup>a</sup>	6.40 $\pm$ 0.57	22.44 $\pm$ 6.95 <sup>a</sup>	8.34 $\pm$ 2.84 <sup>a</sup>
FSH	4	10.52 $\pm$ 2.12 <sup>b</sup>	15.47 $\pm$ 2.19 <sup>a</sup>	16.21 $\pm$ 5.85	2.84 $\pm$ 0.39
LH + FSH	4	16.41 $\pm$ 0.98 <sup>ab</sup>	5.03 $\pm$ 0.12	9.78 $\pm$ 3.59 <sup>b</sup>	6.58 $\pm$ 3.14 <sup>a</sup>

serum concentrations) (Table 3). LH alone as compared with the control increased the production of both testosterone and androstenedione by the testis during both recrudescence and winter dormancy phases, although the increase in androstenedione in dormancy was not statistically significant. FSH alone as compared to the control increased the production of androstenedione but not testosterone by the testis *in vitro* during winter dormancy. During recrudescence, LH caused a significantly higher production of androstenedione as compared with FSH. However, LH and FSH together caused a decline in both androstenedione and testosterone as compared to LH alone. Interestingly, however, during winter dormancy, LH and FSH together, stimulated significantly higher androstenedione but not testosterone production as compared with LH alone.

## Discussion

The cell size and intensity of LH-immunoreactivity of gonadotrophs in the pars distalis of male *S. heathi* showed seasonal variations. However, the weight of the pituitary gland did not vary significantly during most reproductive phases, except during the end of breeding phase when the weight of the pituitary was significantly higher. The seasonal variations in weight of the pituitary gland also showed no significant correlation with testicular weight cycle (Table 1). Among chiropterans, seasonal variations in the population of gonadotroph cells in the male have been reported by Anthony and Gustafson (1984) in *Myotis lucifugus* and by Badwaik (1988) in *Taphozous melanopogon*. The conspicuous hypertrophy and granulation of the LH-cells during October (before the beginning of active spermatogenesis) and early January (the time of mating and beginning of the second peak of spermatogenesis) as noticed in the present study are in accordance with reports on other seasonally breeding mammals such as the ferret (Holmes 1963), vole (Clarke and Forsyth 1964) and the female bat, *Myotis lucifugus lucifugus* (Anthony and Gustafson 1984). A sudden decline in cell size and intensity of LH immunoreactivity has been noticed following the periods of peak spermatogenesis in November and March.



This decrease in pituitary LH-immunoreactivity may suggest increased secretion of LH by the gonadotrophs during the periods of peak spermatogenesis and steroidogenesis. A similar reduction in pituitary LH immunoreactivity has been reported in *M. lucifugus* towards the end of the spermatogenic period (Anthony and Gustafson 1984). Furthermore, degranulation of LH-immunoreactive cells occurs in the periovulatory period in the rat (Blake 1980) and in sows LH-containing gonadotrophs become so extensively degranulated during the week following ovulation that it is difficult to identify them (Herlant and Ectors 1969). The progressive accumulation of LH-immunoreactive material in *S. heathi* was observed from August to October and November to early January. This indicates a higher rate of synthesis than the release of LH from the gonadotrophs during these periods. This suggested that LH is apparently stored in an immunoreactive form within gonadotrophs during winter dormancy as has been shown in female *S. heathi* (Singh and Krishna 1996) and in *M. lucifugus* (Anthony and Gustafson 1984).

During recrudescence in November, *in vitro* studies showed stimulation of both androstenedione and testosterone in response to LH. Increase in the production of both androstenedione and testosterone by the testis in response to LH during winter dormancy in December suggest that the testis contained both receptors and enzymes necessary for steroidogenesis during this period. Therefore, a decline in testicular weight and circulating androstenedione and testosterone level during winter as shown earlier (Krishna and Singh 1997) could be due to a decline in pituitary secretion of gonadotrophin. In female *S. heathi* gonadotrophic activity has also been shown to decline during winter dormancy (Singh and Krishna 1996). However, the much sharper decline in testosterone as compared to only a marginal decline in androstenedione level during winter dormancy in *S. heathi* may be due to increased responsiveness of the testes to melatonin during winter dormancy (Singh and Krishna 1995).

Present *in vitro* studies further demonstrated that along with LH, FSH is also capable of stimulating androgen production from the testis of *S. heathi*. More interestingly, a differential effect of FSH on testicular testosterone and androstenedione production was observed during recrudescence and winter dormancy. During recrudescence, FSH was not very effective in stimulating androstenedione production as compared to the same dose (1  $\mu\text{g/ml}$ ) of LH, whereas during winter dormancy, FSH was found as effective as LH in stimulating androstenedione production. FSH was found comparatively more effective in stimulating testosterone production by the testis *in vitro* during recrudescence than winter dormancy.

Although it is well known that LH is the major stimulus for initiation and maintenance of steroidogenesis by Leydig cells, present studies in the bat along with a number of other studies mainly on the rat, have suggested a vital role for FSH in the control of testicular endocrine functions (Chen *et al.* 1976, Grimek *et al.* 1976, Chandrashekar *et al.* 1994, Gaytan *et al.* 1995). Further, recent studies have shown that treatment with recombinant FSH enhances steroidogenic activities in young hypophysectomized rats (Matikainen *et al.* 1994) and in hypogonadal mice



(O'Shaughnessy *et al.* 1992). Administration of FSH to rat results in an increase in Leydig cell androgen secretion both *in vivo* and *in vitro* (Grimek *et al.* 1976, Chen *et al.* 1976, Odell and Swerdloff 1976). However, a very limited enhancement of testosterone secretion was observed in the Leydig cells cultured with a large dose of FSH in *Suncus murinus* (Furumura *et al.* 1985). The present study further suggests that the rate of testicular androstenedione and testosterone production was higher in response to LH than FSH during recrudescence. This suggests that during recrudescence testicular responsiveness to LH for androgen synthesis was higher than that of FSH. When a testis was incubated together with both LH and FSH much lowered production of androstenedione was observed as compared to the group treated with LH alone. This suggest that FSH can down regulate LH induced testicular androstenedione production during recrudescence. On the contrary, during winter dormancy, FSH and LH together induced a higher production of androstenedione by the testis *in vitro* than by either LH or FSH alone. This suggests upregulation of LH induced androstenedione production by FSH. It has earlier been shown that FSH can regulate the LH-receptor number in the testis (Odell and Swerdloff 1976). But the factor(s) causing this differential modulatory action of FSH during recrudescence and winter dormancy on the ability of LH-induced androstenedione synthesis by the testes of *S. heathi in vitro* is unique and requires further investigation. The present study further suggests changes in FSH responsiveness for testicular androstenedione synthesis from recrudescence to winter dormancy. Higher responsiveness of FSH for testicular androstenedione production during winter dormancy observed in present study may, therefore, be responsible for maintaining high circulating androstenedione concentration in *S. heathi* during this period.

Acknowledgement: We thank NHPP and NIDDK, Baltimore, Md, USA for providing LH and FSH hormone and antisera. The androstenedione antisera was provided by Dr John Resko, Oregon Health Science University, Portland, USA. This study was supported by a grant from UGC, New Delhi, India.

## References

- Abhilasha and Krishna A. 1996. High androgen production by ovarian thecal interstitial cells: a mechanism for delayed ovulation in a tropical vespertilionid bat, *Scotophilus heathi*. *Journal of Reproduction and Fertility* 106: 207–211.
- Anthony E. L. P. and Gustafson A. W. 1984. Seasonal variations in pituitary LH-gonadotrophs of the hibernating bat, *Myotis lucifugus lucifugus*: An immunohistochemical study. *American Journal of Anatomy* 170: 101–115.
- Badwaik N. K. 1988. Cytology and seasonal changes of the pituitary of the emballonurid bat, *Taphozous melanopogon* (Temnick). *Proceeding of Indian Academic of Sciences (Animal Sciences)* 97: 479–489.
- Blake C. A. 1980. Correlative study of changes in the morphology of the LH-gonadotroph and anterior pituitary gland LH secretion during the 4-day rat estrous cycle. *Biology of Reproduction* 23: 1097–1108.
- Chandrashekar V., Majumdar S. S. and Bartke A. 1994. Assessment of the role of follicle-stimulating hormone and prolactin in the control of testicular endocrine function in adult Djungarian hamsters



- (*Phodopus sungorus*) exposed to either short or long photoperiod. *Biology of Reproduction* 50: 82–87.
- Chen Y. D. L., Payne A. H. and Kelch R. P. 1976. FSH stimulation of Leydig cell function in the hypophysectomized immature rat. *Proceeding of Society for Experimental Biology and Medicine* 153: 473–475.
- Clarke J. R. and Forsyth I. A. 1964. Seasonal changes in the adenohipophysis of the role (*Microtus agrestis*). *General and Comparative Endocrinology* 4: 243–252.
- Furumura K., Ota K. and Yokayama A. 1985. Response of interstitial cells of the testes in the house musk shrew, *Suncus murinus* L., to various gonadotrophins. *Theriogenology* 24: 319–325.
- Gaytan F., Bellido C., Morales C., van Rooijen N. and Aguilar E. 1995. Role of testicular macrophages in the response of Leydig cells to gonadotrophins in young hypophysectomized rats. *Journal of Endocrinology* 147: 463–471.
- Grimek H. J., Nuti L. S., Nuti K. M. and McShan W. H. 1976. Effect of neuraminidase treatment on the biological activity of highly purified ovine FSH and LH in hypophysectomized immature male rat. *Endocrinology* 98: 105–110.
- Gustafson A. W. 1979. Male reproductive patterns in hibernating bats. *Journal of Reproduction and Fertility* 56: 317–331.
- Gustafson A. W. 1987. Changes in Leydig cell activity during the annual testicular cycle of the bat (*Myotis lucifugus lucifugus*): Histology and lipid histochemistry. *American Journal of Anatomy* 178: 312–325.
- Herlant M. and Ectors F. 1969. Les cellules gonadotrophes de l'hypophyse chez le porc. *Zeitschrift für Zellforschung und Mikroskopische Anatomie* 101: 212–231.
- Holmes R. L. 1963. Gonadotrophic and thyrotropic cells of the pituitary gland of the ferret. *Journal of Endocrinology* 25: 495–504.
- Krishna A. and Singh K. 1997. The relationship between testicular activity, accessory sex organ and circulating steroid concentration during reproductive cycle of an Indian male vespertilionid bat, *Scotophilus heathi*. *Canadian Journal of Zoology* 75: 1042–1050.
- Matikainen T., Toppari J., Vihko K. K. and Huhtaniemi I. 1994. Effects of recombinant human FSH in immature hypophysectomized male rats: Evidence for Leydig cell-mediated action on spermatogenesis. *Journal of Endocrinology* 141: 449–457.
- Odell W. D. and Swerdloff R. S. 1976. Etiology of sexual maturation: a model system based on the sexually maturing rat. *Recent Progress in Hormonal Research* 32: 245–288.
- O'Shaughnessy P. J., Bennett M. K., Scott I. S. and Charlton H. M. 1992. Effects of FSH on Leydig cell morphology and function in the hypogonadal mouse. *Journal of Endocrinology* 135: 517–525.
- Singh K. and Krishna A. 1995. Inhibitory effects of melatonin on testosterone but not on androstenedione production during winter in the vespertilionid bat, *Scotophilus heathi*. *Journal of Pineal Research* 19(3): 127–132.
- Singh K. and Krishna A. 1996. Seasonal changes in circulating serum concentration and in vitro testicular secretion of testosterone and androstenedione in the male vespertilionid bat, *Scotophilus heathi*. *Journal of Experimental Zoology* 276: 43–52.
- Sternberger L. A., Hardy P. H., Cuculis J. J. and Meyer H. C. 1970. The unlabelled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish-antiperoxidase) and its use in identification of spirochetes. *Journal of Histochemistry and Cytochemistry* 18: 315–333.