

High androgen production by ovarian thecal interstitial cells: a mechanism for delayed ovulation in a tropical vespertilionid bat, *Scotophilus heathi*

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The steroid hormone profile of *Scotophilus heathi*, an Indian tropical vespertilionid bat, was studied, with special reference to the period of delayed ovulation. The results show unusually high concentrations of circulating androstenedione and testosterone during the first half of the period of delayed ovulation, whereas the concentrations of progesterone and oestradiol were relatively low during this period. Androstenedione, testosterone and oestradiol concentrations were high during the period of ovarian recrudescence (October–November). The oestradiol concentration increased, whereas androstenedione and testosterone declined significantly, before ovulation. Studies *in vitro* showed that the ovary secretes more androstenedione and testosterone than does the adrenal. A histological study showed that a major part of the ovary of *S. heathi* was occupied by hypertrophied interstitial cells. An immunocytochemical study showed ovarian thecal interstitial cells as the major site of high androgen synthesis in the ovary. During the period of delayed ovulation the bat ovaries thus resemble the ovaries from women with polycystic ovaries in which abnormally high androgen secretion from hypertrophied interstitial cells prevent ovulation.

Introduction

The phenomenon of delayed ovulation among the temperate zone vespertilionid bats has been known for nearly half a century (Oxberry, 1979). Studies from our laboratory have suggested that *Scotophilus heathi*, a tropical Indian vespertilionid bat, also exhibits this phenomenon (Krishna and Singh, 1992). One of the proposed reasons for the delay in the case of temperate zone bats is the superimposition of the period of hibernation on the season of reproduction (Oxberry, 1979; Racey, 1982). The primary aim of this study was to investigate the hormonal changes in *S. heathi* during the period of delayed ovulation. The data presented in this study indicate that high concentrations of circulating androgens may be responsible for suppression of ovulation, as found in patients with polycystic ovary syndrome (PCOS). Therefore, an attempt was made to determine whether the adrenal gland or the ovary is the major source of high androgen secretion in *S. heathi* and to localize the site of high androgen synthesis in the ovary.

Part of this work was presented at the 27th Annual Meeting of the Society for the Study of Reproduction, held in Ann Arbor, MI, USA.

Materials and Methods

Bats were captured live every fourth week from Banaras Hindu University Campus and adjacent areas. They were killed by decapitation as soon as they were brought to the laboratory. Blood serum was collected (pooled blood from two bats) and

stored at -20°C until assayed for androstenedione, testosterone, oestradiol and progesterone. On the basis of the known reproductive cycle of *S. heathi* (Krishna and Singh, 1992), females were classified into the following phases:

- (1) Quiescence: a reproductively inactive phase, during which the ovary does not contain any antral follicles (August–September).
- (2) Recrudescence: the beginning of reproductive activity when the ovaries contain some newly formed antral follicles (October–November).
- (3) Early phase of ovulatory delay during which there is a temporary arrest in reproductive activity. Ovaries contain some antral follicles (December–early January).
- (4) Late phase of ovulatory delay: the ovaries contain antral follicles and mating occurs (mid-January–mid-February).
- (5) Preovulatory period during which there is renewed ovarian activity and the ovaries contain large antral follicles (late February–early March).

Steroid radioimmunoassay

The circulating concentrations of steroids were measured by radioimmunoassay according to the method of Krishna and Terranova (1987). The antibody for androstenedione was obtained from J. Resko (Oregon Health Science University, Portland, OR), and progesterone, oestradiol and testosterone antisera were purchased from ICN, Biomedical Inc. (Costa Mesa, CA). Radioactive androstenedione was purchased from Amersham (Bucks) and oestradiol, progesterone and testosterone from ICN Biomedical Inc. Steroids in the serum

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samples were extracted with 2 ml diethyl ether; the ether extract was decanted, evaporated to dryness at 37°C and resuspended in 0.01 mol phosphate buffer saline–gelatin ml⁻¹ (PBSG) for further analysis. The *in vitro* media were assayed without extraction.

Studies in vitro

The *in vitro* study was performed to determine whether the ovary or the adrenal gland is the major source of high circulating androgens in *S. heathi*. Bats collected during early December were killed as soon as they were brought to the laboratory. Their ovaries and adrenals were dissected out immediately and cleaned in Media 199 (Gibco). Ovaries (one per tube) were incubated with 1.0 µg LH (NIADDK ovine LH-26) and adrenals (one per tube) with 1 µg adrenocorticotrophic hormone (ACTH) (Sigma) in 1 ml media-199 with 0.1% (w/v) BSA for 2 h at 37°C. Samples of media were frozen after the incubation at -20°C until assayed for androstenedione and testosterone.

Histology

Ovaries from bats were sectioned serially and stained with haematoxylin and eosin.

Immunocytochemistry

Ovaries collected during November were used for immunocytochemistry. Steroidogenic enzymes, P₄₅₀ 17α hydroxylase (P₄₅₀ 17α-OH) and P₄₅₀ side chain cleavage (P₄₅₀ SCC) were localized using the peroxidase antiperoxidase (PAP) technique. The primary antibodies against rat P₄₅₀ 17α-OH and rat P₄₅₀ SCC were obtained from A. H. Payne (University of Michigan, Ann Arbor, MI) and M. J. Soares (Kansas Medical Center, Kansas City, KS), respectively. For the secondary antibody and PAP, a rabbit immunostaining kit (No. 30313324, Zymed Laboratories, San Francisco, CA) was used. Paraffin wax sections were deparaffinized in xylene, and hydrated through graded ethanol. The endogenous peroxidase activity was blocked by treating sections with 3% (v/v) H₂O₂ in 10% (v/v) methanol for 20 min. Slides were rinsed with PBS and incubated for 30 min in 10% (v/v) normal goat serum to eliminate nonspecific binding. The slides were then incubated with primary antibody (rabbit anti-rat P₄₅₀ 17α-OH, 1:200 dilution; rabbit anti-rat P₄₅₀ SCC, 1:200 dilution) in a moist chamber for 4 h. After rinsing in PBS, sections were incubated with goat anti-rabbit IgG for 30 min. Again, the slides were rinsed with PBS and incubated with rabbit PAP solution. After washing in Tris–HCl saline buffer (0.05 mol l⁻¹), the sections were developed in 0.01% (w/v) 3'-3' diaminobenzidine tetrahydrochloride and 0.01% (v/v) H₂O₂ in Tris–HCl buffer, dehydrated and mounted in distreine pathalate xylene (DPX). All the incubations were carried out at room temperature. The control slides were incubated with buffer instead of primary antibody.

Electron microscopy

Bats (*n* = 3) collected during December were killed by perfusion fixation. One-hundred iu heparin (Liquemin 100R,

Hoffmann La Roche, Grenzach-Whylem, Germany) per 100 g body mass were injected i.p. under anaesthesia (6 mg pentobarbital per 100 g body mass), 15 min before beginning the perfusion. The thoracic and abdominal cavities were opened along the midline of the body and a blunt cannula fitted to a silicone tube was introduced into the left ventricle and slowly pushed into the ascending aorta. After fixing the cannula with one clamp, and interrupting the large cervical blood vessels with other clamps, the remaining blood circulation was rinsed by 50 ml Ringer's solution using a perfusor. The speed of the perfusor was adjusted to 300 ml h⁻¹. After rinsing, 50 ml fixative, 1% (v/v) glutaraldehyde, 1% (w/v) paraformaldehyde in 0.1 mol Sorensen's buffer l⁻¹ (pH 7.4), was injected. The ovaries were dissected out and trimmed pieces of ovary were postfixed in 2% (v/v) osmium tetroxide. Tissues were embedded in Araldite.

Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference and Schiffe's Test when differences were significant. Differences were considered significant when *P* < 0.05. Data are expressed as means ± SEM.

Results

Circulating concentrations of steroids in relation to delayed ovulation

Circulating concentrations of androstenedione, testosterone, oestradiol and progesterone during different stages of ovarian cycles are shown in Fig. 1. The concentration of androstenedione began to increase from quiescence and reached an unusually high peak during recrudescence. The concentration of testosterone also began to increase from quiescence and was high during recrudescence, reaching a peak during the early phase of ovulatory delay. Serum oestradiol concentration increased, whereas progesterone concentration decreased from quiescence to recrudescence. Concentrations of testosterone and androstenedione were high during the early phase of ovulatory delay, whereas oestradiol concentration fell during this phase. Serum concentration of both androstenedione and testosterone gradually declined from the early to the late phase of ovulatory delay, when mating takes place, and reached a minimum during the preovulatory phase. The oestradiol concentration increased before ovulation. Progesterone concentrations in serum were unchanged during the period from recrudescence until before ovulation.

The major source of high circulating androgen

The concentration of androstenedione and testosterone secreted *in vitro* by the ovary and the adrenal following stimulation with LH and ACTH, respectively, are shown in Fig. 2. The ovary secreted a far larger amount of both androstenedione and testosterone compared with the adrenal.

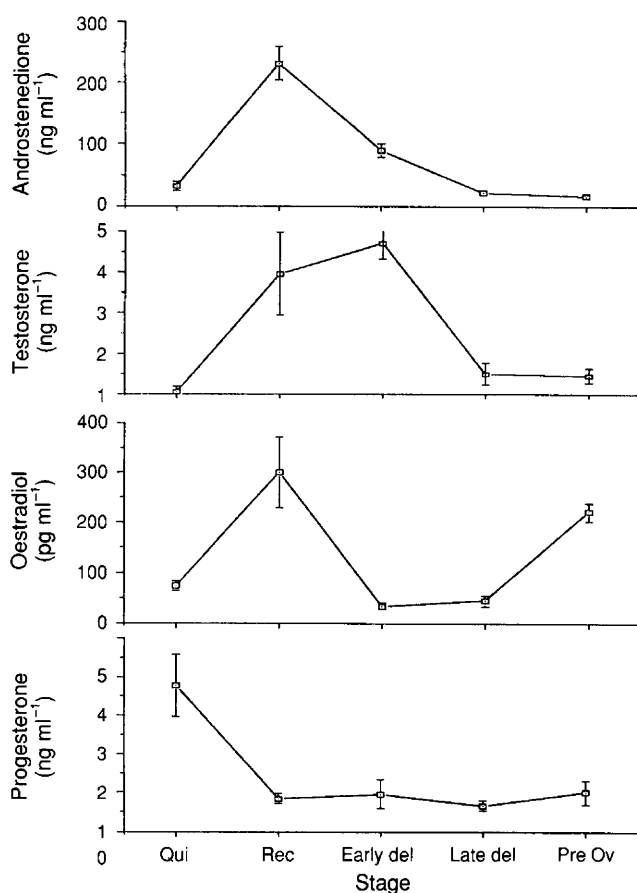


Fig. 1. Variation in serum concentrations of androstenedione, testosterone, oestradiol and progesterone in *Scotophilus heathi* during different reproductive stages. Values are means \pm SEM. Qui: quiescence ($n = 12$; 15 August 1992); Rec: recrudescence ($n = 8$; 3 November 1992); Early del: early phase of ovulatory delay ($n = 10$; 7 December 1992); Late del: late phase of ovulatory delay ($n = 8$; 8 January 1993); Pre Ov: preovulatory period ($n = 6$; 28 February 1993). Values in parentheses indicate the number of bats (n) used and the time of collection.

The site of androgen synthesis in the ovary

Histology. The ovary was relatively large in November–December and contained a number of follicles of different sizes, both healthy and atretic, separated by the extensive interstitial tissues. The interstitial cells were so extensive that, besides the follicles, they occupied the major part of the ovary (Fig. 3a). Interstitial cells were polygonal with comparatively smaller nuclei and a highly vacuolated cytoplasm (Fig. 3b). They were found associated with healthy as well as atretic follicles. Under the microscope, thecal cells and interstitial cells appeared similar (Fig. 3c). Ultrastructural studies on interstitial cells revealed abundant round to ovoid lipid droplets (Fig. 3d). Rod to round-shaped mitochondria were distributed between lipid droplets and constituted the second most conspicuous cytoplasmic organelle. The endoplasmic reticulum was generally agranular and ribosomes were dispersed throughout the cytoplasm.

Immunocytochemistry. Immunocytochemical localization of steroidogenic enzymes P₄₅₀ SCC and P₄₅₀ 17 α -OH showed

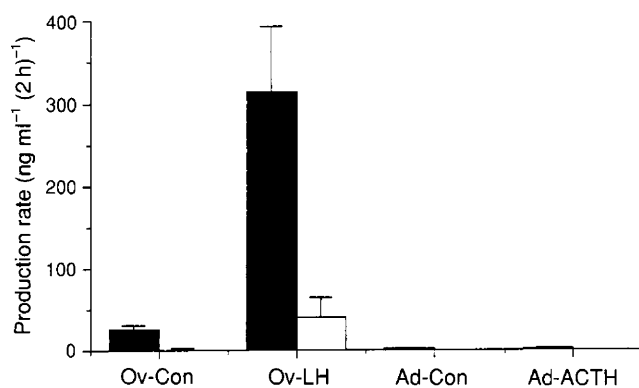


Fig. 2. Effect of LH on ovarian and ACTH on adrenal androstenedione (■) and testosterone (□) production *in vitro* during early December (7 December 1992) in *Scotophilus heathi*. Values are means \pm SEM. Ov-Con: ovary control ($n = 3$); Ov-LH: ovary LH-stimulated ($n = 3$); Ad-Con: adrenal control ($n = 4$); Ad-ACTH: adrenal ACTH-stimulated ($n = 4$). n : number of tissues incubated.

intense positive reaction, mainly in the thecal cells and interstitial cells of the ovary (Fig. 3e, f). Granulosa cells did not show any positive immunoreaction. Control slides showed no reaction.

Discussion

The phenomenon of delayed ovulation found in temperate zone vespertilionid bats is thought to be related in some way to the metabolic depression characteristic of the hibernating state (Oxberry, 1979; Racey, 1982). Although none of the tropical vespertilionids undergoes prolonged hibernation, many seem to resemble hibernating bats in having a reproductive cycle characterized by slow follicular maturation, unique specialization in the Graafian follicle and a prolonged oestrus (Dominic and Krishna, 1989). Thus, it appears that the prolonged hibernation, which characterizes temperate zone species, is not a necessary preadaptation for delayed ovulation. To our knowledge, this is the first detailed study describing the changes in circulating steroid concentrations in relation to delayed ovulation in a vespertilionid bat. The results indicate that the ovary of *S. heathi* secretes unusually large amounts of both androstenedione and testosterone during the period of recrudescence and the early phase of ovulatory delay; circulating concentrations decline to very low values during the period when mating and ovulation normally take place in this species (Krishna and Singh, 1992). Thus, it is possible that the high circulating concentrations of androstenedione and testosterone may be responsible, in part, for suppressing or delaying ovulation in *S. heathi*.

Female *S. heathi* in the present study displayed concentrations of circulating androstenedione that were unusually high compared with the values reported for most other mammals (Feder, 1985; Glickman *et al.*, 1992). The concentrations of circulating testosterone were also high, but not as high as those of circulating androstenedione. To our knowledge the circulating androstenedione and testosterone concentrations of any other female bat are not known. However, whenever plasma testosterone concentrations have been

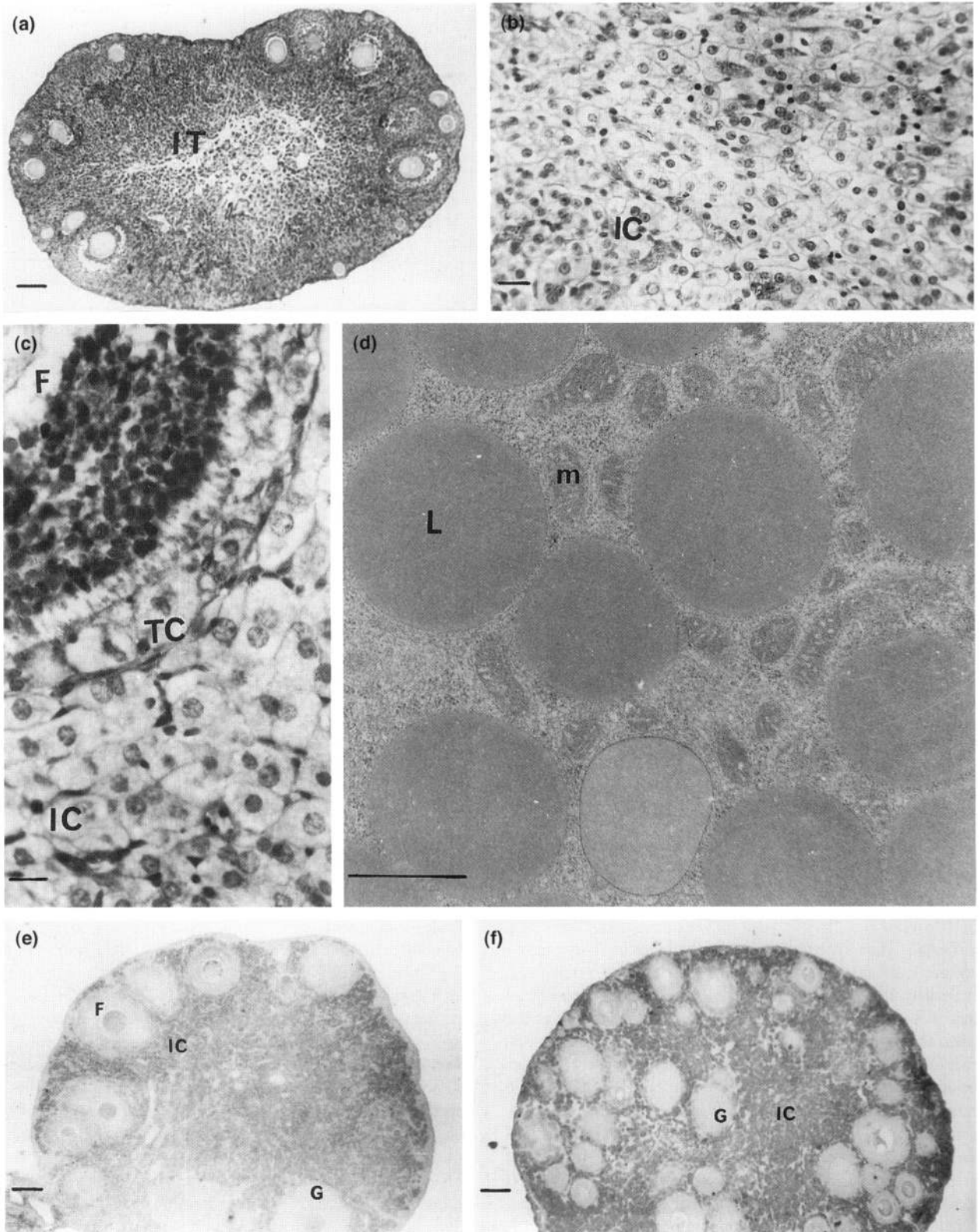


Fig. 3. (a) Cross-section of the ovary of *Scotophilus heathi*, showing extensive interstitial tissue (IT) and a number of growing follicles in the periphery. Scale bar represents 100 μm . (b) Interstitial tissue as seen under light microscope at high magnification. Interstitial cells (IC) with vacuolated cytoplasm and an irregular nucleus are seen. Scale bar represents 50 μm . (c) A portion of the ovary showing a part of the follicle (F). Note the similarity between thecal (TC) and interstitial cells (IC). Scale bar represents 10 μm . (d) Fine structure of interstitial cell, showing a large number of lipid droplets (L) of various sizes and mitochondria (m). Scale bar represents 1 μm . (e) Immunocytochemistry of P_{450} side chain cleavage in the ovary of *S. heathi*. The thecal cells and interstitial cells (IC) are immunostained. The granulosa cells (G) are negative. F: follicle. Scale bar represents 100 μm . (f) Immunocytochemistry of P_{450} 17 α -OH in the ovary of *S. heathi*. The thecal cells and interstitial cells (IC) are immunostained. The granulosa cells (G) are negative. Scale bar represents 100 μm .

measured in male microchiropterans, the majority have been extremely high compared with those of most eutherians (Racey, 1974; Gustafson and Shemesh, 1976).

The present study *in vitro* indicates that the ovary is the major source of both androstenedione and testosterone in *S. heathi*. One of the most striking cytological features of the ovary of *S. heathi* is the dominance of the interstitial cells. Practically all of the interfollicular tissue of the ovary of *S. heathi* consisted of a mass of interstitial cells. Histologically, the thecal cells and interstitial cells looked alike and, therefore, may be called thecal-interstitial cells. Thecal-interstitial cells are generally formed owing to accumulation of thecal cells from the atretic follicle in the stroma (Guraya and Greenwald, 1968). Although such extensive development of interstitial cells has been reported in some species of bat (Kayanja and Mutere, 1975; Rasweiler, 1988; Tsvetkov and Takeva, 1988), its significance has not yet been demonstrated. Immunocytochemical localization of P₄₅₀ SCC and P₄₅₀ 17 α -OH in the ovary suggests that thecal-interstitial cells are the major site of steroidogenic activity and androgen secretion. Overproduction of androgen is therefore attributed to the hypertrophied thecal-interstitial cells of the ovary in *S. heathi*.

The intraovarian effects of androgens are well documented in rodents and include changes in steroid production, inhibition of follicular growth and ovulation and an enhanced rate of follicular atresia (Louvet *et al.*, 1975; Lindner *et al.*, 1977; Azzolin and Saiduddin, 1983). Androgens have also been implicated aetiologically in the establishment of PCOS in humans (Yen, 1980). It has been shown experimentally in rats and monkeys that treatment with high concentrations of androgen results in morphological changes in the ovary, resembling those found in PCOS (Ward *et al.*, 1978; Billiar *et al.*, 1985; Faiman *et al.*, 1988). Polycystic ovaries are characterized by large numbers of small antral follicles surrounded by hypertrophied thecal and interstitial cells; these cells produce large amounts of androgens which prevent the follicles from ovulating (Rosenfield *et al.*, 1990). One of the most important consequences of PCOS is the failure to produce dominant follicles in the ovary. Similarly, in *S. heathi* thecal-interstitial cells surrounding the follicles showed intense P₄₅₀ SCC and P₄₅₀ 17 α -OH activities, and thus may be secreting large amounts of androgen. Oversecretion of androgen may have inhibited follicular growth and, consequently, selection of a dominant follicle. Singh and Krishna (1992) failed to induce ovulation in this species using hCG and pregnant mares' serum gonadotrophin during the period of ovulatory delay. This study also indicates the absence of dominant or selected follicles in this species during this period. The causes of the decline in serum androgen concentrations during the preovulatory period are not known. However, further research may prove useful for developing *S. heathi* as a model to investigate the mechanism responsible for hyperandrogenism and to devise therapeutic strategies for its treatment.

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