# Immunolocalization of cytochrome $P_{450}$ side chain cleavage, 17-$\alpha$-hydroxylase and aromatase in the ovary of vespertilionid bat (*Scotophilus heathi*) during different phases of ovulatory delay

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*Immunocytochemical localization of steroidogenic enzymes, cytochrome $P_{450}$ side chain cleavage, 17-α-hydroxylase and aromatase, was performed in the ovaries of* ***Scotophilus heathi*** *during reproductive cycle, with reference to the period of delayed ovulation. Moderate immunoreactivity of side chain cleavage enzyme and 17-α-hydroxylase was observed mainly in thecal cells and interstitial cells of the ovarian stroma during quiescence. Thecal cells positive for 17-α-hydroxylase were found even around the primary follicles. The peak immunoreactivity for all the three enzymes was observed during recrudescence. It coincided with high circulating steroid levels during this period. In the stroma, immunoreactivity for side chain cleavage and 17-α-hydroxylase was so extensive that it almost occupied the entire interfollicular area of the ovary. Aromatase immunoreactivity declined, but side chain cleavage enzyme and 17-α-hydroxylase remained extensive during the period of delayed ovulation. This suggests a high androgen and low estrogen synthesis during the period of delayed ovulation. There was a marked decline in 17-α-hydroxylase and an increase in aromatase immunoreactivity during the preovulatory period, suggesting a decrease in androgen and increase in estrogen synthesis. The results suggest thecal cells and interstitial cells of the stroma as the major site of steroidogenesis in the ovary of* ***S. heathi****. Over production of androgen is attributed to the extensive development of 17-α-hydroxylase positive interstitial cells in the ovarian stroma, and this may be responsible for delayed ovulation in* ***Scotophilus heathi****.*

***Key-terms:*** *aromatase, bat, cytochrome $P_{450}$, delayed ovulation, 17-α-hydroxylase, ovary, side chain cleavage enzyme*

## INTRODUCTION

The bats have long since fascinated biologists because they exhibit various forms of reproductive delays. Four types of reproductive delay patterns have been noted in chiropterans: delayed ovulation, delayed fertilization, delayed implantation and delayed development (Bernard, 1989). Although the phenomenon of delayed ovulation, postponement of final maturation and ovulation of Graafian follicle, are known among vespertilionid bats for nearly half a century, the specific reason for the postponement of ovulation in this bat is not clearly known. *Scotophilus*

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*heathi* also exhibit delayed ovulation and factors responsible for this were examined recently (Abhilasha & Krishna, 1996). Our study suggested that abnormally high androgen secretion from hypertrophied stroma of the ovary prevents ovulation in this species (Abhilasha & Krishna, 1996). A similar kind of association between hyperandrogenism and anovulation is well known in the patients with polycystic ovarian disease (PCOD) (Rosenfield *et al*, 1990). Over expression of ovarian cytochrome $P_{450}$ 17-α-hydroxylase (17-OH) was shown to be responsible for hyperandrogenism in most of the cases of PCOD (Rosenfield *et al*, 1990). According to Erickson *et al* (1989), the primary events leading to infertility (anovulation) in PCOD involved defects in estrogen ($E_2$) biosynthesis, specifically the aromatization of androstenedione (A) to $E_2$. Axelrod and Goldzieher (1961) failed to detect aromatase activity in PCOD. We have recently characterized the steroid hormone profile of *S. heathi* during different stages of ovulatory delay (Abhilasha & Krishna, 1996). Our findings have suggested unusually high levels of A and a comparatively low level of $E_2$ in circulation, during the period of delayed ovulation, similar to that reported in PCOD. Interestingly, the plasma levels of $E_2$ increased whereas A level declined significantly before ovulation in this bat. Similar preovulatory changes occur in normal ovulatory cycle of mammals. This interesting hormonal pattern has generated a quest for exploring the expression and distribution patterns of steroidogenic enzymes in the ovary of *S. heathi* during different stages of ovulatory cycle.

In the ovarian steroidogenic pathway, cytochrome $P_{450}$ cholesterol side chain cleavage (SCC), $P_{450}$ 17-α-hydroxylase (17-OH) and $P_{450}$ aromatase (AROM) are important regulatory enzymes which catalyze the conversion of cholesterol to pregnenolone, pregnenolone to androgens and androgens to $E_2$, respectively. In the present study, cellular localization and intensity patterns of these enzymes in the ovary of *S. heathi* during different stages of ovulatory cycle was investigated by immunocytochemistry and results are reported here.

## MATERIALS AND METHODS

All the bats (n=24) were trapped alive from Banaras Hindu University campus and adjacent areas. Reproductive cycle of *S. heathi* has been classified into the following phases (Krishna & Singh, 1992):

1. **Quiescence**: reproductively inactive phase, ovary does not contain any antral follicle (August-September).

2. **Recrudescence**: beginning of follicular development, ovary contains some newly formed antral follicles (October-November).

3. **Winter Dormancy (Period of ovulatory delay)**: it is the phase of temporary arrest of reproductive activity, ovary contains some antral follicles (December-mid February).

4. **Preovulatory period**: period of renewed ovarian activity, ovary contains large antral follicles (late February-early March).

Bats from each phase were sacrificed by decapitation and their blood serum was collected. Ovaries were excised out from the body cavity and weighed separately. Ovaries were immediately fixed in Bouin's solution, and embedded in paraffin wax according to commonly used histological techniques.

### *Immunocytochemistry*

Steroidogenic enzymes, SCC, 17-OH and AROM, were localized using the peroxidase anti-peroxidase (PAP) technique. The primary antibody against rat SCC, rat 17-OH and human recombinant AROM, were obtained from Dr Soares (Kansas City, USA), Dr Payne (Ann Arbor, USA) and Dr Johnson (Kansas City, USA), respectively. For secondary antibody and PAP, rabbit immunostaining kit (Lot No. 30313324) of

Zymed Laboratory (USA) was used. Paraffin sections were deparaffinized in xylene and hydrated through ethanol grade. To block the endogenous peroxidase activity, sections were treated with 3% $H_2O_2$ in 10% methanol for 20 minutes. Slides were rinsed with PBS and incubated for 30 minutes in 10% normal goat serum to eliminate non-specific binding. The slides were incubated with primary antibody (rabbit anti-rat SCC, 1:200 dilution; rabbit anti-rat 17-OH, 1:200 dilution and rabbit anti-human AROM, 1:200 dilution), in a moist chamber for 4 hrs. After rinsing with PBS, sections were incubated with goat anti rabbit IgG (1:100) for 30 minutes. Again the slides were rinsed with PBS and incubated with rabbit PAP (1:50) solution. After washing in Tris-HCL saline buffer (0.05 M, TBS) the sections were developed in 0.01% 3-3'diaminobenzidine tetrahydrochloride (DAB) and 0.01% $H_2O_2$ in TBS, dehydrated and mounted in DPX. All the above incubations were carried out at room temperature. For immunostain controls, normal rabbit IgG or PBS were used instead of the primary antibodies. Immunocytochemistry for each enzyme in the ovarian sections from different phases were processed together in a single run. Every 20th serial ovarian section from at least three bats was used for immunocytochemical analysis of each enzyme during each phase.

### *Steroid radioimmunoassay*

Steroids (A, $E_2$ and progesterone) were determined by radioimmunoassay as described in detail earlier (Abhilasha & Krishna, 1996).

## RESULTS

Seasonal changes in the serum steroid (A, $E_2$ and progesterone) concentration during different phases of reproductive cycle are summarized in Table I.

Immunoreactivity of steroidogenic enzymes (SCC, 17-OH and AROM) in the ovary (Table II) shows seasonal variations. During the **quiescence phase,** moderate immunoreactivity of SCC and 17-OH was observed mainly in the thecal cells (TCs) and ICs of the ovarian stroma (Fig 1a,b). In the stroma, more intense reaction was observed in the cortical region (peripheral) as compared to the medullary region (central) of the ovary. TCs of all the growing follicles showed positive reaction. Even in the primary follicles, 1-2 layers of theca-like cells showed positive reaction for 17-OH. The number of concentric layers of ICs and/or TCs around each follicle showing intense positive immunoreactivity for 17-OH increases with the stages of development (Table III, Fig 1d,e). Granulosa cells (GCs) showed only a faint staining for 17-OH but staining was negative for SCC. Oocytes of most of the follicles also showed mild immunoreaction for both SCC and 17-OH (Fig 1a,b). The staining in oocytes was generally darker in follicles in early stages of development. AROM showed mild reaction in the ICs during this phase.

The intensity of SCC, 17-OH and AROM immunoreactivity was maximum during **recrudescence** (Fig 1f-h). Difference in the

**Table I**

Seasonal variations in serum level of androstenedione, estradiol 17β and progesterone of *S. heathi.*

| | August | November | Early January | Late February | P (ANOVA) |
|---|---|---|---|---|---|
| Androstenedione (ng/ml) | 29.9 ± 6.8 | 162.7 ± 30.5 | 64.6 ± 0.3 | 17.3 ± 0.8 | < 0.001 |
| Estradiol 17-β (pg/ml) | 98.1 ± 23.3 | 278.9 ± 109.8 | 56.97 ± 12.0 | 202.3 ± 43.0 | < 0.001 |
| Progesterone (ng/ml) | 3.4 ± 1.0 | 1.3 ± 0.4 | 1.0 ± 0.3 | 1.0 ± 0.4 | < 0.01 |

Data are means ± SE of three samples. Serum samples are pooled from two bats.

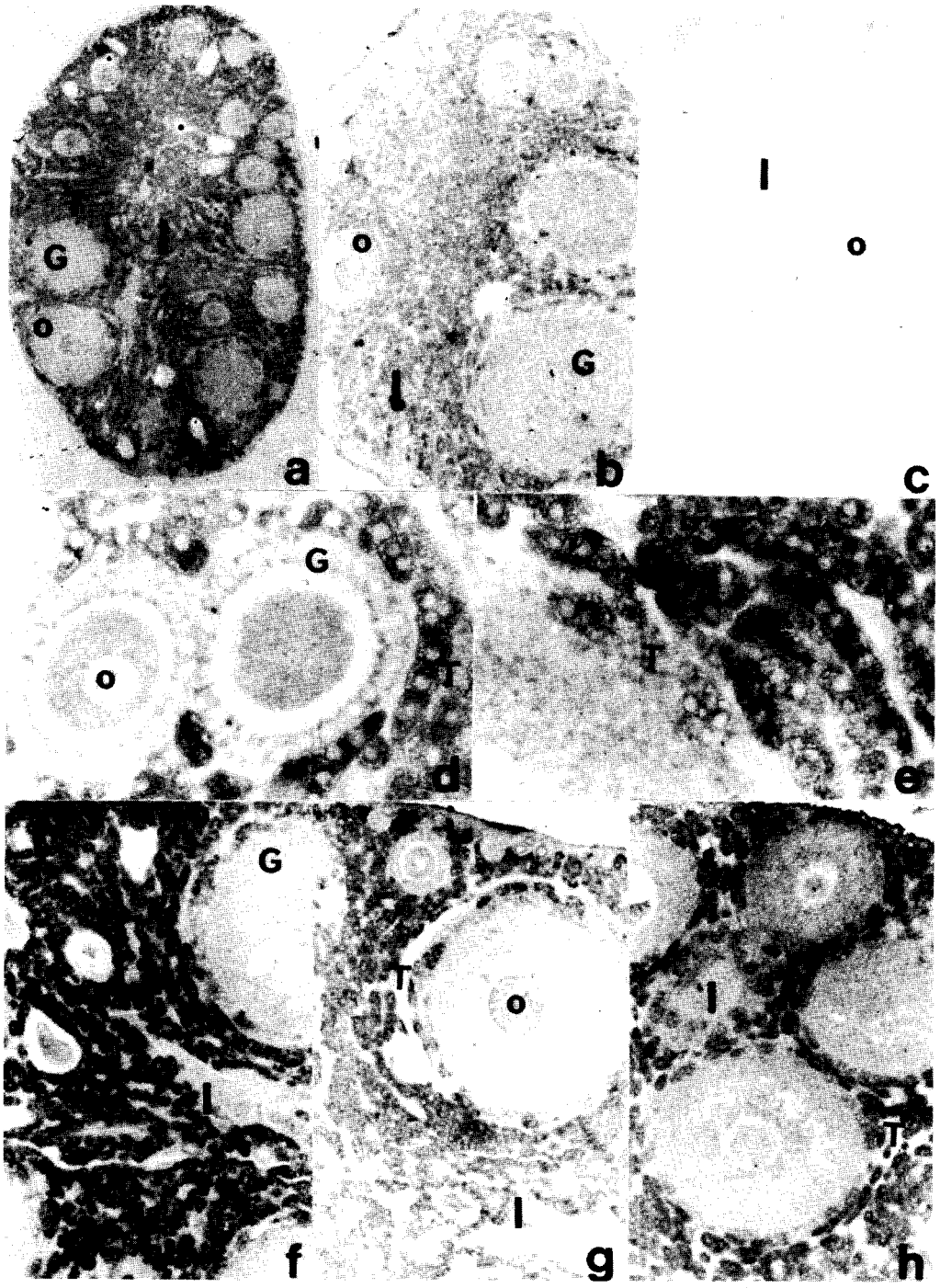


**Fig 1.** Immunolocalization of steroidogenic enzymes in the ovarian sections of *S. heathi*. **a**. SCC during quiescence. x 30. **b**. 17-OH enzymes during quiescence. x 30. The immunoreactivity is present mainly in thecal cells and interstitial cells. Immunoreactivity is also present in the oocytes (broken arrow) and regressing follicle (arrow). **c**. Control section showing no positive immunoreactivity. x 60. **d**. Primary follicle (containing 1 or 2 layers of GCs showing one layer of TCs/ICs arranged around the follicle (arrow). x 250. **e**. A portion of antral follicle showing 4-5 concentric layers of 17-OH immunoreactive TCs/ICs. Some cells of the innermost layer of theca interna are not showing any immunoreactivity (arrow). x 250. **f-g**. Immunoreactivity during recrudescence. Reaction present mainly in Tcs and Ics. Granulosa cells show immunoreactivity for AROM only. G, granulosa cells; I, interstitial cells; O, oocyte; T, thecal cells. **f**. SCC immunoreactivity during recrudescence. x 67. **g**. 17-OH immunoreactivity during recrudescence. x 67. **h**. AROM immunoreactivity during recrudescence. x 67.

**Table II**

Seasonal variation in immunoreaction (intensity and distribution) of steroidogenic enzymes in the ovary of *S. heathi*

| | SCC | | | | 17-OH | | | | AROM | | | | P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | O | GC | TC | IC | O | GC | TC | IC | O | GC | TC | IC | < |
| Qui | 0.67 | 0.67 | 1.67 | 2.33 | 1.00 | 0.67 | 2.00 | 2.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 |
| Rec | 0.33 | 1.33 | 3.00 | 3.67 | 0.00 | 0.67 | 2.67 | 4.00 | 1.00 | 1.33 | 1.67 | 3.44 | 0.01 |
| Del ov | 0.00 | 0.00 | 2.33 | 2.33 | 0.00 | 0.00 | 1.67 | 2.67 | 0.00 | 0.00 | 0.00 | 1.33 | 0.01 |
| Pre ov | 1.00 | 0.00 | 1.00 | 3.00 | 0.00 | 0.00 | 1.00 | 1.00 | 0.00 | 0.00 | 2.33 | 2.67 | 0.01 |
| P < | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | |

Scores given as follows: absence of immunoreactivity = 0; mild = 1; moderate = 2; intense = 3; very intense = 4. Data are means of scores from 3 bats. Every 20th serial sections of the ovary from each bat was used for observation. Non-parametric ANOVA calculated by Kruskal-Wallis test was found significant ($p<0.01$) when compared stage wise (vertically) or cell type-wise (horizontally). P-values are the same for each enzyme group. **Qui** = Quiescence; **Rec** = Recrudescence; **Del ov** = Delayed ovulation; **Pre ov** = Preovulatory period.

intensity of the reaction between cortical and medullary region was not as prominent as in the quiescence. At this stage very intense immunostaining of SCC and 17-OH was located mainly in the TCs and ICs of the stroma. The number of ICs showing intense immunoreactivity for these enzymes was increased. In the stroma SCC and 17-OH immunoreactivity was so extensive that it occupied almost all the interfollicular area of the ovary. Here also, the number of concentric layers of ICs and/ or TCs showing positive reaction for SCC and 17-OH increases with stages of follicular development (Table III). GCs showed only a mild immunoreactivity for SCC and 17-OH. AROM immunoreactivity was intense in the ICs, whereas only a moderate immunoreaction was observed in the GCs and TCs. Mild immunoreactivity for AROM was also seen in the oocytes, during this period. Immunoreactivity of SCC and 17-OH was retained in the TCs of the follicles undergoing atresia.

**Table III**

Number of concentric layers of 17-OH immunopositive TCs/ICs surrounding different type of follicles in the ovary of *S. heathi* during various phases of reproduction.

| | Primary | Preantral | Antral | P < |
|---|---|---|---|---|
| Quiescence | 1-2 (1.33) | 3-4 (3.33) | — | 0.01 |
| Recrudescence | 1-2 (1.67) | 3 (3.00) | 4-5 (4.67) | 0.01 |
| Delayed Ovulation | 1-2 (1.33) | 2 (2.00) | 4 (4.00) | 0.01 |
| Preovulatory | — | 2-3 (2.67) | 4-5 (4.67) | 0.01 |
| P | < 0.01 | < 0.01 | > 0.05 | |

All follicles with oocyte present on every 20th serial sections were counted. Data showing range of immunopositive concentric layer. Data within brackets, means of three bats. P-value calculated by Kruskal-Wallis tests. **Primary** = follicle containing 1-2 layers of granulosa cells. **Preantral** = follicle with more than 2 layers of granulosa cells. **Antral** = follicle with antral spaces.

During the **period of ovulatory delay,** the intensity of immunoreactivity for all the three enzymes declined as compared to recrudescence. However, the number of TCs and ICs showing SCC and 17-OH immunoreactivity remained as extensive as observed during recrudescence. Mild immunoreactivity for AROM was observed only in the ICs. No immunoreaction for any of the enzymes was observed in the GCs and oocytes.

During **preovulatory period,** marked decline in the intensity of 17-OH immunoreactivity was observed. Many of the ICs showed mild or no immunoreactivity for 17-OH. The extent of 17-OH immunoreactivity in the TCs and ICs also showed a marked decline. Most of the cells in the theca interna layers, lying directly beneath the membrana granulosa did not show any immunoreactivity. SCC immunoreactivity remained intense and extensive in ICs but mild in TCs. The intensity of AROM immunoreactivity was significantly increased in the TCs and ICs.

## DISCUSSION

The female reproductive cycle of *S. heathi* exhibits unique the phenomenon of delayed ovulation (Abhilasha & Krishna, 1992). Earlier studies in this species clearly demonstrated that the abnormally high circulating androgen level during the period of recrudescence and winter dormancy may be responsible for delayed ovulation (Abhilasha & Krishna, 1996). The present study provides a detailed investigation on the immunocytochemical localization of steroidogenic enzymes (SCC, 17-OH and AROM) in the ovarian compartments of *S. heathi*, during different phases of reproductive cycle and is comparable with circulating steroid concentration reported earlier from our laboratory (Abhilasha & Krishna, 1996). The results indicate no evidence of any block in steroidogenic pathway except a suppression of AROM activity during the period of ovulatory delay. Rather, very intense immunoreaction for steroidogenic enzymes, SCC and 17-OH was observed in the ICs and TCs during the periods of recrudescence and delayed ovulation. Presence of intense SCC and 17-OH and low AROM may be the reason for over secretion of A. The results reported here, thus confirm the recent report of Abhilasha and Krishna (1996), that delayed ovulation in *S. heathi* represents a condition of hyperandrogenism.

Similar to the rat (Roby *et al*, 1991) and various other species studied (Sasano *et al*, 1989), SCC immunoreactivity in the ovary of *S. heathi* were mainly localized in the TCs of the follicle and ICs in the stroma. Absence of immunoreactivity in the GCs in the present study is in accordance with previous reports of low levels of SCC in GCs of other species (Rodgers *et al*, 1986). Only during the recrudescence GCs of some antral follicles showed mild immunoreactivity. Thus this study suggests that $C_{21}$ steroid biosynthesis may occur primarily outside the GC compartment. SCC also showed seasonal variations in the intensity of immunoreactivity during the ovarian cycle (Table II). The peak SCC immunoreactivity in the ovary of *S. heathi* during the recrudescence coincides with the peak circulating level of steroids shown in this species (Abhilasha & Krishna, 1996).

The amount of 17-OH enzyme immunoreactivity reflects the capacity of the ovary to produce androgen from $C_{21}$ steroids. Similar to SCC, 17-OH activity in the ovary was localized mainly in the TCs of the follicles and ICs of the stroma. The number of TCs layers showing 17-OH immunoreactivity increases with the follicular development and growth. Even the TCs around the primary or early secondary follicles (Fig 1d) showed immunoreactivity for 17-OH. In the human ovary, 17-OH immunoreactivity was shown in the theca interna of the antral follicles (Tamura *et al*, 1992); however, no report is available about the preantral follicles. The absence of immunoreactivity for 17-OH in one to several layers of theca interna cells located just beneath the membrana granulosa in the large healthy antral follicles was unique. These theca interna cells may not be involved in excess A synthesis and follicles containing this type

of cells may have advantage in the selection for further development. More importantly, the 17-OH immunoreactivity was found to be prominently intense in the ICs of the stroma specially during the periods of recrudescence and delayed ovulation. Previously, the 17-OH was localized essentially in the TCs of bovine, porcine, rat and human ovaries, but its localization in the stroma was inconsistent (Sasano *et al*, 1989). However, when this steroidogenic enzyme was evaluated in the polycystic ovaries with stromal hyperthecosis, a similar intense immunostaining was observed in the luteinized stroma (Erickson *et al*, 1985).

Immunocytochemistry of AROM revealed the presence of immunoreactivity mainly in the TCs and ICs. Only a mild immunoreactivity was observed in the GCs, specially during recrudescence. Similar pattern of AROM localization, mainly in the TCs was earlier shown in human polycystic ovary (Tamura *et al*, 1993). Seasonal variations in the AROM immunoreactivity seems to be partially responsible for the abnormal hormonal pattern shown in *S. heathi*. Low concentration of $E_2$ found during delayed ovulation correlated well with the suppressed AROM immunoreactivity. Further, high A concentration observed during this period may be due to the inappropriate AROM activity and consequent accumulation of substrate. The factor(s) causing the decline in AROM during the period of delayed ovulation is not known. However, this supra normal amount of A with simultaneous low $E_2$, causing high A : $E_2$ ratio is similar to what others have observed in the cases of PCOD and anovulation (Erickson *et al*, 1989). In PCOD inappropriate FSH release causes suppression of AROM (Barnes, 1991). Exposure to excessive androgen appears to enhance the rate of follicular atresia through the inhibition of AROM activity (Azzolin & Saiduddin 1983). Furthermore, the decline in A concentration observed during preovulatory period may be due to the increase in AROM and decrease in 17-OH immunoreactivity.

The present study also showed the localization of SCC, 17-OH and AROM immunoreactivity in the oocytes and suggested that oocytes of the bat may produce androgen and $E_2$. Suzuki *et al* (1984) have shown the presence of $E_2$ in the oocytes, while Nekola and Nalbandov (1971) have suggested that oocyte secretes factors which inhibit luteinization of GCs. It is assumed that A and/or $E_2$ produced from oocytes may be necessary in the early stages of follicular and oocyte development.

The presence of morphologically differentiated and 17-OH positive theca-like cells around the developing follicles containing only 1 or 2 layers of GCs, is an interesting observation. Morphological studies of rodent ovary have shown that definite thecal layers appear only when follicles contain 5-6 layers of GCs (Roy & Greenwald, 1985). In most of the mammals, the theca interna cells (TICs) of developing late secondary and small antral follicles express little or no 17-OH indicating that during the early stages of differentiation, the TICs are devoid of steroidogenic activity or produce primarily $C_{21}$ steroid (Roy, 1994). The presence of theca like 17-OH positive cells around the primary or early secondary follicles implies that in this species TCs may not be formed from undifferentiated prethecoblast cells but derived from the pool of already differentiated theca-like ICs from the stroma. This is supported by the observation that both ICs and TCs were identical in morphology and immunoreactivity for the enzymes SCC and 17-OH. This hypothesis was further supported by the observation that the maximum number of 17-OH positive ICs in the ovarian stroma was observed before the beginning of active follicular development in the recrudescence. The 17-OH positive ICs are gradually being used up as the follicular development proceeds and ultimately a lowest level of 17-OH immunoreactive ICs was observed in the ovarian stroma during the preovulatory period (Table II). Several ICs, almost emptied of 17-OH immunoreactivity were observed during the preovulatory period. This may be the reason for high androgen production during the recrudescence and early phase of ovulatory delay but decline during preovulatory period. It has so far

been not known that TIC differentiation can occur independently of follicular development. It is intriguing to know, how the extensive ICs are developed in this species before the recrudescence. The present study further indicates that the number of 17-OH positive TIC layers around the developing follicle increases with the stages of the follicular development (Table III). This is because the ICs organized around the developing follicles in response to some factors released from oocytes and/or GCs. It has been shown that tumor necrosis factor α or some other growth factors such as epidermal growth factor may function as thecal organizing factor, that attracts these ICs from the stroma to the oocyte during the follicular development (Zachow *et al*, 1992; Erickson *et al*, 1985).

The ovary of *S. heathi* during recrudescence and winter dormancy exhibited intense 17-OH immunoreactivity in practically entire interfollicular stroma, which consisted of a mass of theca-like ICs. Over production of androgen is therefore attributed to the extensive development of 17-OH positive ICs in the ovary of *S. heathi*. Such an extensive development of ICs has earlier been reported in some species of bats (Kayanja & Muture, 1975; Rasweiler, 1988; Tsvetkov & Takeva, 1988). The ovary of female spotted hyenas with masculinization of external genitalia also showed hypertrophied ICs as the major source of androgen secretion (Lindeque *et al*, 1986). Similarly increased mass of theca-like ICs in the ovarian stroma was shown to be the main site of excess androgen production in PCOD (Erickson *et al*, 1985). Further studies are in progress to investigate the factor(s) causing such an extensive development of 17-OH positive ICs in the ovarian stroma of *S. heathi*. This information might be helpful in revealing the mysteries about the pathogenesis of PCOD.

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