Adiposity associated rise in leptin impairs ovarian activity during winter dormancy in Vespertilionid bat, *Scotophilus heathi*

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**Abstract**

The aim of the study was to evaluate the seasonal variation in serum leptin levels in a natural population of the female bat, *Scotophilus heathi* and their relationship to the changes in the body mass, serum insulin level, and ovarian activity. Circulating leptin level varied significantly over the season and correlated positively with the changes in body mass, and circulating insulin and androstenedione (A4) levels. Circulating leptin concentrations showed two peaks; one coincides with the maximum fat accumulation prior to winter dormancy, whereas the second shorter peak coincides with late pregnancy. The *in vivo* study in *S. heathi* showed that the increased circulating leptin level during winter dormancy coincides with the decreased expression of ovarian steroidogenic acute regulatory (StAR) protein, and low circulating estradiol (E2) level. At the same time, increased circulating leptin level coincides with increased expression of ovarian insulin receptor and high circulating A4 level. The *in vitro* study confirmed the *in vivo* observations of inhibitory effect of leptin on LH induced StAR expression and E2 production, whereas the stimulatory effect of leptin (high dose) on LH induced expression of insulin receptor protein and A4 production. However, pharmacological dose of leptin produced inhibitory effect on the expression of insulin receptor protein. The results of the present study thus suggest that high circulating leptin level during winter dormancy promotes adiposity and impairs ovarian activity by suppressing StAR-mediated E2 production as well as by enhancing insulin receptor-mediated A4 synthesis thereby contributing anovulatory condition of delayed ovulation in *S. heathi*.

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**Introduction**

*Scotophilus heathi*, a vespertilionid bat, breeds once in a year (seasonally monoestrous) from March to July and exhibits a unique phenomenon of delayed ovulation (Krishna & Singh 1992). During the reproductive cycle of *S. heathi* the ovary undergoes recrudescence in October resulting in development of large antral follicles. The ovary of *S. heathi* showed the presence of large antral follicles from October onwards, but ovulation is delayed until March. Attempts to induce ovulation during this period were unsuccessful (Singh & Krishna 1992). This suggests that the ovarian activity in *S. heathi* is impaired during the period of delayed ovulation from November to January, which also coincides with the period of winter dormancy and fat accumulation. The ovary of *S. heathi* synthesized androstenedione (A4) was at a high concentration during the period from November to January and was shown to be responsible for the suppression of follicular maturation and delayed ovulation in this bat species. Further, studies on *S. heathi* demonstrated a significant correlation between circulating A4 concentration with the increase in body mass due to the accumulation of adipose tissue (adiposity) (Abhilasha & Krishna 1997). Based on these studies, a close association between adiposity, increased androgen synthesis and anovulation was suggested in *S. heathi* as documented in women with polycystic ovary syndrome (PCOS) (DeWailly et al. 1998). On the basis of these findings, it was suggested that *S. heathi* might be used as an animal model for PCOS. The obesity factors, which may be responsible for increased A4 synthesis during the period of delayed ovulation in *S. heathi*, are under investigation in our laboratory (Doval & Krishna 1998, Chanda et al. 2003).

Available evidences implicate the role of at least two peripheral hormones, leptin, and insulin, as providing key afferent information to the CN (CNS) or peripheral tissues concerning the amount and distribution of body fat (Benoit et al. 2004). The anabolic actions of insulin on peripheral tissues are well established and plasma insulin also apparently serves as a signal of body fat content to the CNS (Schwartz et al. 1992). Insulin also amplifies the lipogenesis in adipose tissue. The metabolic hormone insulin has been known as the major
physiological regulator of energy balance in mammals (Boswell et al. 1994). A rise in the circulating insulin concentration after enhanced eating has been reported (Cosgrove & Foxcroft 1996). It has also been reported that a deficiency or an excess of insulin could significantly alter ovarian functions, including folliculogenesis and steroidogenesis (Stuart et al. 1986). Hyperinsulinemia (HI) is present in young girls undergoing puberty (Nobels & DeWailly 1992). Diabetic girls do not complete puberty unless they receive adequate insulin replacement (Bergquist 1954). This suggests that insulin has a role in the hormonal changes associated with obesity as well as with the ovulatory process. Studies on S. heathi also showed a close relationship between circulating insulin concentration and changes in the body mass. Numerous studies demonstrate the co-existence of hyperandrogenism (HA) and elevated insulin level (Sam & Dunaif 2003). Insulin is also shown to directly affect the ovarian steroidogenesis in S. heathi (Doval & Krishna 1998). The study on S. heathi further showed the increased circulating concentration of insulin coinciding with increased A4 level during the period of delayed ovulation. Both insulin and A4 level declined significantly during the preovulatory period suggesting a significant correlation between circulating insulin and A4 concentrations in S. heathi (Doval & Krishna 1998). Further, study on the bat showed a linear relationship between adiposity (body mass), insulin, and A4 in the bat as reported in women with the PCOS (Doval & Krishna 1998). Increase in serum A4 concentration during the period of weight gain (September–November) and a decline in serum A4 concentration during the period of weight loss (December–February) in S. heathi suggests that changes in the adiposity correlates closely with gonadal steroidogenesis in this species. Thus, investigation on factors mediating adiposity-associated variation on ovarian steroidogenesis may provide information about the mechanism of delayed ovulation in S. heathi; it may also provide some clues about the etiology of PCOS.

Leptin, the hormone product of the obesity (ob) gene (Zhang et al. 1994) is synthesized predominantly in adipose tissue (Masuzaki et al. 1995) and its expression and release in rodents is stimulated by insulin (Saladin et al. 1995, Leroy et al. 1996). In humans, serum leptin levels are highly correlated with the percentage body fat and fall in response to weight loss (Maffei et al. 1995, Considine et al. 1996, Weigle et al. 1997). Increasing adiposity is accompanied by insulin resistance (IR) and compensatory HI (Kopelman 1994), and suggests the possibility of an interaction between insulin and leptin. Severe dietary restriction, as noticed during winter dormancy, catabolic states, and even short-term caloric deprivation, impair fertility in mammals. Likewise, obesity is associated with infertile condition such as in the PCOS. Fertility seems to require the integration of reproduction and metabolic signals. Leptin appears to be the important factor between fat and fertility. Until recently, insulin has been assumed to be the major link between nutrition and reproduction (Schneider et al. 2000). Clearly, many of the phenomena that have been attributed to insulin have to be re-investigated in relation to leptin. Although other factors such as prolactin, melatonin, and thyroxine are also known to play important roles in seasonal reproduction, in the present study, however, the interaction between insulin and leptin in regulation of ovarian activity are specifically investigated in the bat species.

The role of leptin in reproduction includes its actions on the hypothalamus to bring about the release of gonadotropin leading to development of the reproductive tract and induction of puberty (Caro et al. 1996). Administration of leptin to obese leptin-deficient mutant mice increased ovarian activity and restoration of fertility (Barash et al. 1996, Chehab et al. 1996, Kikuchi et al. 2001). A direct involvement of leptin in ovarian function has been postulated following the demonstration of leptin-receptors in the ovaries of numerous mammalian species (Karlsson et al. 1997, Ruiz-Cortés et al. 2000, Kikuchi et al. 2001). The majorities of studies have suggested that the direct effects of leptin on ovarian cells are inhibitory and can be attributed to attenuation of steroidogenesis (Zachow & Magoffin 1997, Zachow et al. 1999, Duggal et al. 2000, Spicer et al. 2000, Ghizzoni et al. 2001, Guo et al. 2001). Little is known about the genes targeted by leptin in the ovary. Given the known influences of leptin on steroid synthesis, the steroidogenic acute regulatory (StAR) protein and P450 side chain cleavage (SCC) enzyme are the important candidates for leptin regulation. StAR and SCC are the key elements in the rate-limiting steps of steroid biosynthesis. StAR regulates cholesterol delivery to the P450 SCC enzyme located in inner mitochondrial membrane, which consequently converts cholesterol into pregnenolone (Clark et al. 1994, Stocco & Clark 1996). Effects of leptin on luteinizing hormone (LH) and insulin receptors in the ovary are also not studied. Most of the studies on role of the leptin in reproduction have been conducted on rodents in artificial settings.

Therefore, the aim of this study was to evaluate the seasonal variation in serum leptin level in a natural population of the female bat, S. heathi and its relationship to the changes in body mass, serum insulin level and ovarian activity particularly with reference to steroid synthesis. Since, leptin is known to modulate insulin activities and vice versa, this led us to investigate the effect of leptin on ovarian expression of insulin receptor protein in S. heathi.

Materials and Methods

All of the experiments were conducted in accordance with principles and procedures approved by the Banaras Hindu University Departmental Research Committee.
Bats were captured alive every 2–4 weeks from Banaras Hindu University, Varanasi (25°N, 83°E) and adjacent areas. Details of study site and feeding activity were described earlier in detail (Singh & Krishna 1996). In Varanasi, the cold season lasts from November to February (mean ambient temperature < 20 °C) and dry season from March to June (mean ambient temperature > 30 °C). During the cold season, the evening temperature is often too low (6–9 °C) to permit foraging. S. heathi exhibits winter dormancy or torpidity accompanied by decreased activity from mid-December to January. During this period, they exhibit hypothermia and their anal temperature was also low (24–28 °C; ambient temperature < 20 °C) as compared to other (30–40 °C; ambient temperature > 20 °C) periods of the year. They also stop feeding (no foraging) during the winter as observed from their gut content. Bats were generally trapped during each phase of the reproductive cycle between 0800 and 1000 h directly from their natural habitat. Bats were usually euthanized by decapitation as soon as they were taken to the laboratory. To minimize the stress, body mass, which include the weight of the decapitated head from the bat, and other details were recorded after the bat was killed. Weight of accumulated adipose tissue in S. heathi during different months was adopted from the earlier study (Chanda et al. 2003). The white adipose tissue accumulated under the skin was carefully separated out (using a scalpel and scissors) from the bat euthanized in the glass jar pretreated with the overdose of anesthetic ether. Serum was collected from blood samples (pooled blood from two bats) within one hour and stored at −20 °C until assayed for leptin, insulin, and androstenedione. Ovaries were cleaned and kept frozen at −20 °C until the immunoblots were performed.

S. heathi has a sharply defined annual reproductive cycle and can be classified into the following phases (Krishna & Abhilasha 2000): (1) Recrudescence (October): beginning of reproductive activity; (2) First Wave of Follicular Development (Early November): ovaries contain newly formed antral follicles; (3) Winter dormancy & Period of delayed ovulation (Late November-January): bats remain torpid showing temporary arrest in reproductive activity, ovaries contain some antral follicles; (4) Second Wave of Follicular Development (February): ovaries contain newly formed antral follicles; (5) Preovulatory period (Early March): ovaries contain large antral follicles. Females are pregnant from March to July; lactating during July and quiescent during August–September.

In vivo study
This in vivo study was undertaken to assess the effect of seasonal increase and decrease in leptin secretion on ovarian activity, particularly in reference to steroid synthesis in S. heathi. The ovarian activity was assessed by determining the changes in the expression of P450 SCC enzyme, StAR protein, LH-receptor and insulin receptor in bats collected during different reproductive stages. Circulating A4 and 17 β estradiol (E2) levels were determined to assess ovarian steroidogenesis.

In vitro study
The in vitro study was performed on the ovaries collected during January to determine the effects of leptin (high and pharmacological doses) and insulin on LH-induced steroid (A4, Progesterone & E2) synthesis as well as expression of insulin receptor and StAR protein in the ovaries of S. heathi. The effect of leptin and insulin was compared with the effect of LH alone. The dose of LH and insulin was determined from our earlier studies (Doval & Krishna 1998, Krishna & Abhilasha 2000). The two doses, high and pharmacological, for leptin were adopted from earlier studies (Ruiz-Cortés et al. 2003). Female S. heathi were sacrificed by decapitation as soon as they were brought to the laboratory. Their ovaries were quickly taken out and cleaned for any adhered fat tissue and oviduct in medium Dulbecco Modified Eagle’s Medium (DMEM; Himedia, Mumbai, India) containing 250 U/ml penicillin and 250 μg/ml streptomycin sulfate. Ovaries were cultured by the method as described previously (Mayerhofer et al. 1997) with some modifications. Culture medium was a mixture of DMEM (with sodium pyruvate and L-glutamine) and Ham’s F-12 (1:1; v:v) (Himedia, Mumbai, India) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1% BSA (Sigma). After initial incubation for 2 h at 37 °C, culture medium was discarded and ovaries (one per tube) were finally cultured in 1 ml medium in a humidified atmosphere with 95% air and 5% CO2 to maintain pH 7.4 for 48 h at 37 °C with either leptin (100 ng/ml or 1 μg/ml) or insulin (200 ng/ml) with LH (100 ng/ml). Each treatment group was run in triplicate. Ovaries cultured under these conditions appear healthy and do not show any sign of necrosis. Ovaries were collected at the end of culture, washed several times with PBS and kept frozen at −20 °C for immunoblot study. Media was saved at −20 °C until assayed for A4, progesterone (P), and E2.

Hormone assay
Leptin
The circulating concentration of leptin in the female S. heathi was measured by RIA using a multi-species leptin RIA kit obtained from Linco Research Inc., St Louis, MO, USA. The leptin kit was validated for use in S. heathi (Fig. 1). Blood serum sample from bats (n = 6) were pooled and serially diluted with assay buffer and tested against a standard curve. Dilutions of bat serum ran parallel to standard curve indicating the suitability of the assay for use in S. heathi. Assay was performed in accordance with the
instructions provided by the manufacturers. Bound radioactivity was measured for one minute in Beckman Gamma Counter. Standards, zero standard and blank tubes were also processed along with the samples. Intra-assay coefficient of variation was <8%.

**Insulin**

Insulin was measured by RIA using a human kit from Medicorp, Montreal, Canada. The protocol was used as provided by manufacturer. Briefly, 100 µl serum sample was added to each insulin antibody coated tube. To each tube, 1 ml 125I-insulin was added and incubated for 18 h at room temperature. Finally, decanted liquid from each assay tube except total count tubes and counted for 1 min in Beckman gamma counter. Standards, zero tubes and blank tubes were also run along with the samples. Intra-assay coefficient of variation was less than 8.2%. Validation of insulin assay for *S. heathi* was described earlier (Doval & Krishna 1998).

**Androstenedione, progesterone and estradiol**

The serum steroids were measured after extraction, whereas steroids in the culture medium were measured directly using RIA kits. Serum samples for A4 (25 µl), and E2 (200 µl) were diluted to 1 ml with distilled water and extracted twice with 2 ml anhydrous diethyl ether. The aqueous phase was frozen, and organic (solvent) phase was removed and evaporated to dryness at 37 °C and resuspended in 0.01 M phosphate buffer saline gelatin for further analysis (Abhilasha & Krishna 1996). Antibody-bound steroids were separated from free steroids by the addition of 500 µl dextran-coated charcoal solution and centrifugation at 1700 g. Supernatant (500 µl) was then mixed with 5 ml scintillation cocktail and counted in LKB Wallace liquid scintillation counter. The antibodies used in the present study were highly specific and showed <0.01% cross reactivity with either sulfate or glucuronate bound steroids or other steroids (estradiol-17α, pregnenolone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, testosterone, dihydroepiandrostenedione (DHEA), ethiocholanolone). Extraction efficiency was determined and the average recovery was 92.5%. Steroids in the culture medium were measured by RIA using a human kit from Immunotech, Marseille, and France. Assays for A4, P, and E2 in the culture medium were performed with 25, 100, and 100 µl of the medium respectively, as per instructions provided by the manufacturer. Control serum, standard, zero tubes, and blank tubes were run in parallel with the samples. Intra-assay coefficient of variation for all the assays were less than 12%. Steroid assays for *S. heathi* were validated earlier (Abhilasha & Krishna 1996).

**Immunoblot**

Three ovaries were pooled to produce 10% homogenate. Further, protein extraction and immunoblot was performed as described previously (Chanda et al. 2004). Equal amount of proteins (50 µg) as determined by Folin’s method was loaded on to SDS-PAGE (10%) for electrophoresis. Thereafter, proteins were transferred electrophoretically to nitrocellulose membranes (Sigma-Aldrich) overnight at 4 °C. Nitrocellulose membranes were blocked for 60 min with Tris-buffered saline (TBS; Tris 50 mM (pH 7.5), NaCl 150 mM) containing 5% fat-free dry milk and incubated with insulin receptor β antibody (at a dilution of 1:1000), rabbit anti-human P450 SCC antibody (at a dilution of 1:1000), rabbit anti-human LH receptor antibody (at a dilution of 1:100) and rabbit anti-human STAR antibody (at a dilution of 1:2000) for one hour at room temperature. Antibodies against LH receptor and P450 SCC enzyme were generously supplied by Craig S. Atwood (Texas Tech University, Health Sciences Center, Lubbock, TX, USA) and antibody directed against StAR was kindly provided by D M Stocco (Texas Tech University, Health Sciences Center, Lubbock, TX, USA) and antibody against insulin receptor β subunit was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Membranes were then washed with three changes of TBS over 10 min. Immunodetection was performed with anti-rabbit IgG-alkanine phosphatase conjugate (1:2000 dilution in TBS) except for the LH receptor. Finally, we washed the blot three times with TBS and developed with enhanced chemiluminescence (ECL) detection system (Vector laboratories, Burlingame, CA, USA). Immunoblot for LH receptor were performed by the same method except that they were detected with anti-rabbit IgG-horse radish peroxidase antibody (at a dilution of 1:500) using diamieno-benzidine tetrahydrochloride (DAB) as substrate (Tris–Cl 100 mM)

Figure 1 Correlation between pooled serum samples from *S. heathi* and a standard curve. The coefficient of determination (r²) for the bat serum samples versus standard curve B/B₀ was 0.99.
(pH 7.6), DAB 0.06%, H₂O₂ 0.03%). Immunoreactive bands were later quantified by densitometry (Quantity one software, Bio-Rad). Immunoblots for StAR protein were performed in the same blot, probed for insulin receptor, after washing with stripping solution (Tris 62.5 mM (pH 7.0), SDS 2%, 2-mercaptoethanol 0.7%) for 30 min at 60 °C with continuous shaking in a water bath. This was followed by the three subsequent washings with TBS-Tween (0.02% v/v Tween-20 in TBS (pH 7.5)). Membranes were treated as fresh and reprobed for StAR protein with rabbit polyclonal anti-human StAR antibody (at a dilution of 1:2000 in blocking solutions) by the method described above. Experiments were repeated thrice with the same result. Equal loading was confirmed with Ponceau S staining.

**Statistical analysis**

The seasonal changes in body mass, serum insulin, and leptin concentration were analyzed by ANOVA followed by Duncan’s test. Student’s t-test and correlation study were performed to compare the data from different groups.

**Results**

**Changes in body mass, fat content and ovarian weight**

Monthly changes in the body mass and ovarian mass of female bat are shown in Table 1. Changes in the body mass of female bat was marked with gain in body mass before winter dormancy (November) due to the gradual accumulation of adipose tissue reaching maximum level, about 50% above the basal level, as a result of overfeeding. Fat deposition in this species is preceded by a period of increased insect availability (August–October) and consumption (Krishna 1982). White adipose tissue mainly accumulated subcutaneously in the neck, back, lateral abdominal, and pelvic regions. The adipose tissues were gradually metabolized during winter dormancy (December–January) resulting in loss of body mass with basal level attained in February. Females also showed an increase in body mass during June due to increase in fetal mass (Table 1).

**Changes in circulating leptin concentration and its correlation with body mass and circulating insulin and androstenedione concentration: an in vivo study**

Monthly changes in serum leptin and insulin concentrations are shown in the Fig. 2. Serum leptin concentration exhibited two peaks, one in December and another in June which is similar to insulin. The leptin level remained high during winter period from November to January and then again during late pregnancy in June. Leptin level remained low prior to ovulation and during lactation. Insulin exhibited two peaks, one in December prior to winter dormancy and another in June during late pregnancy. The two peaks of insulin coincided with the seasonal peaks of serum leptin concentration. A significant decline in serum insulin concentration was observed prior to ovulation in March. Monthly changes in the serum androstenedione and estradiol levels are described earlier in detail (Krishna & Abhilasha 2000).

Serum leptin concentration correlated positively with the changes in the body mass (Fig. 3A; r = 0.80; P < 0.05) and the serum insulin (Fig. 3B; r = 0.57; P < 0.05) and androstenedione (Fig. 3C; r = 0.48; P < 0.05) concentrations. The serum insulin concentration correlated positively with the changes in body mass (Fig. 3D; r = 0.66; P<0.05) and androstenedione (r = 0.56; P<0.05).

**Correlation of circulating leptin concentration with ovarian expression of luteinizing hormone receptor, insulin receptor, P450 SCC and StAR**

Western blot analysis of total insulin receptor in the ovaries of *S. heathi* showed a single immunoreactive band at 97 kDa (Fig. 4). Densitometric analysis of

<table>
<thead>
<tr>
<th>Calendar months</th>
<th>Body mass (g)</th>
<th>Ovarian mass (mg)</th>
<th>White adipose tissue (g)</th>
<th>Estradiol (pg/ml)</th>
<th>Androstenedione (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>August</td>
<td>30.8±0.2</td>
<td>13.8±0.1</td>
<td>0.9±0.1</td>
<td>102.8±21.7</td>
<td>36.8±5.6</td>
</tr>
<tr>
<td>September</td>
<td>30.8±0.4</td>
<td>12.6±0.3</td>
<td>0.9±0.1</td>
<td>128.3±59.9</td>
<td>57.8±14.1</td>
</tr>
<tr>
<td>October</td>
<td>33.0±0.7</td>
<td>15.7±0.1</td>
<td>3.9±0.4</td>
<td>66.5±9.4</td>
<td>85.7±18.3</td>
</tr>
<tr>
<td>November</td>
<td>45.5±0.5</td>
<td>18.5±0.1</td>
<td>11.4±0.8</td>
<td>556.5±133.6</td>
<td>220.5±50.1</td>
</tr>
<tr>
<td>December</td>
<td>42.3±1.1</td>
<td>14.2±0.4</td>
<td>4.6±0.2</td>
<td>197.9±123.7</td>
<td>86.3±15.1</td>
</tr>
<tr>
<td>January</td>
<td>37.7±0.9</td>
<td>13.4±0.3</td>
<td>2.6±0.3</td>
<td>51.9±12.8</td>
<td>21.8±2.1</td>
</tr>
<tr>
<td>February</td>
<td>31.8±0.8</td>
<td>11.8±0.3</td>
<td>2.6±0.3</td>
<td>227.0±28.0</td>
<td>16.6±1.3</td>
</tr>
<tr>
<td>March</td>
<td>33.5±0.3</td>
<td>11.6±0.4</td>
<td>2.6±0.3</td>
<td>137.2±35.9</td>
<td>119.2±24.4</td>
</tr>
<tr>
<td>April</td>
<td>32.1±0.6</td>
<td>11.5±0.3</td>
<td>2.6±0.3</td>
<td>147.1±54.5</td>
<td>161.5±19.0</td>
</tr>
<tr>
<td>May</td>
<td>36.4±0.6</td>
<td>11.5±0.3</td>
<td>2.6±0.3</td>
<td>91.0±27.4</td>
<td>146.5±25.9</td>
</tr>
<tr>
<td>June</td>
<td>43.3±0.7</td>
<td>11.5±0.3</td>
<td>2.6±0.3</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>July</td>
<td>30.8±0.5</td>
<td>11.5±0.3</td>
<td>2.6±0.3</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. of 6–8 samples.

aNegligible (<0.5 g). bCould not be determined due to the limited number of animals captured during the period of matured pregnancy and abortion.
Western blot of insulin receptor during the period from October to March showed a marked variation. The immunoreactivity of insulin receptor remained low during the months of October (recrudescence) and November (follicular development I), but increased markedly to a high level during the months of December (early delayed) and January (late delayed). The expression of insulin receptor protein declined substantially during February (follicular development II). Western blot analysis of LH-receptor in the ovaries of *S. heathi* showed a single immunoreactive band at 70 kDa (Fig. 4). Densitometric analysis of western blot of LH receptor during the period from October to March showed only a limited variation. StAR protein in the ovaries of *S. heathi* showed a single immunoreactive band at 30 kDa (Fig. 4). Densitometric analysis of western blot of StAR during the period from October to March showed a marked variation. The expression of StAR protein showed a peak during October coinciding with the ovarian recrudescence. The StAR protein level decreased during November–December and reached its lowest level in January. The StAR protein expression increased again during February–March, coinciding with the preovulatory changes in the ovary. The expression of ovarian P450 SCC showed only a marginal variation during the period from October to March. The expression of ovarian P450 SCC protein in the ovaries of *S. heathi* showed two immunoreactive bands at 49 and 42 kDa (Fig. 4).

**Effects of leptin and insulin on ovarian steroidogenesis and expression of insulin receptor and StAR proteins in vitro**

The effects of leptin (high and pharmacological doses) and insulin on LH-induced steroid (A4, P & E2) synthesis by *S. heathi* oocytes *in vitro* are shown in Figs 5 and 6. Both high and pharmacological doses of leptin enhanced LH-induced ovarian production of A4 and P *in vitro* during January. Insulin, at both the doses,
enhanced LH-induced A4 production but only lower dose enhanced LH-induced ovarian production of P in vitro. Pharmacological dose of leptin decreased LH-induced production of E2 in vitro. However, the lower dose of leptin produced no significant changes in LH-induced E2 synthesis.

Both low dose of leptin and insulin enhanced LH-induced expression of insulin receptor protein, but suppressed the expression of StAR protein, in the ovaries of S. heathi (Fig. 7). Pharmacological doses of leptin, however, suppressed the expression of both insulin receptor and StAR protein.

Discussion
The results of the present study demonstrate a significant positive relationship between circulating leptin concentration and body mass in a free-living vespertilionid bat, S. heathi. Such a relationship between the circulating leptin level and the body mass index (BMI) or percent body fat has earlier been demonstrated in rodents and human (Maffei et al. 1995). In human and rodents, high leptin level correlates with high BMI increased energy

Figure 4 (A) Western blot analysis of insulin receptor protein in the ovaries of S. heathi during the reproductive cycle. (B) The blot was used again for immunodetection of StAR protein in the ovaries of S. heathi during the reproductive cycle. (C) Western blots analysis of LH receptor protein and P450 SCC enzyme in the ovaries of S. heathi during the reproductive cycle. A nonspecific band at 42 kDa was found in the immunoblot for P450 SCC enzyme.

Figure 5 Effect of high (H) and pharmacological (P) dose of leptin and insulin on LH (100 ng/ml) stimulated androstenedione and progesterone production by the ovaries of S. heathi in vitro. Leptin: high dose = 100 ng/ml, pharmacological dose = 1 µg/ml; Insulin: high dose = 200 ng/ml, pharmacological dose = 1 µg/ml. Values are mean ± S.E.M., n = 6. *Values are significantly different (P<0.05) than the LH treated group. Each bar represents mean ± S.E.M.

Figure 6 Effect of high (H, 100 ng/ml) and pharmacological (P, 1 µg/ml) dose of leptin, on LH (100 ng/ml) stimulated estradiol production by the ovaries of S. heathi in vitro. *Values are significantly different (P<0.05) than the LH treated group. Each bar represents mean ± S.E.M.

Figure 7 Western blot analyses of StAR and insulin receptor proteins in the ovaries of S. heathi treated in vitro with Leptin and Insulin together with LH. Treatment with LH only served as control. The same blot was used for immunodetection of both StAR and insulin receptor protein. The different lanes in the above mentioned blots represent (1) control (LH only, 100 ng/ml) (2) LH (100 ng/ml) + insulin (200 ng/ml) (3) LH (100 ng/ml) + leptin (100 ng/ml) (4) LH (100 ng/ml) + insulin (1 µg/ml) (5) LH (100 ng/ml) + leptin (1 µg/ml).

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consumption and decreased feeding behavior (Friedman 1997). In other vespertilionid bat, Myotis lucifugus, plasma leptin level decreased during late pre-hibernating period when adiposity was highest (Kronfeld-Schor et al. 2000). Dissociation between leptin level and body adiposity, as seen in M. lucifugus, was not observed in the present study in S. heathi. The difference in the pattern of circulating leptin levels observed in the two vespertilionid bats during pre-hibernating period may be attributed to difference in the physiological action of leptin. In S. heathi, the plasma leptin level increases in November as the body mass increases due to accumulation of white adipose tissue. The concentration of leptin attains highest level at the beginning of winter dormancy in December when the fat content is also highest. Fat deposition in this species is preceded by a period of increased insect availability (August–October) and consumption (Krishna 1982). The plasma leptin level in S. heathi decreases in January–February when the body mass declined due to metabolism of fat. Since a high level of circulating leptin was found at the beginning of adipose tissue accumulation, it is likely that leptin may be a signal for adipocytes differentiation from preadipocytes, although further studies are required to confirm this. Furthermore, fat stored in S. heathi may be necessary for the bat's survival throughout winter when insects are in short supply. A similar association between leptin and fat mass during winter is shown in other mammals (Hissa et al. 1998). These changes reflect that leptin is one of the most important factors associated with the seasonal changes in the fat accumulation in S. heathi and may provide a reliable index of body fat.

The present study showed two peaks of circulating leptin concentration during the annual cycle of S. heathi. The higher peak of leptin in December coincides with the peak adiposity, whereas the relatively shorter second peak in June coincides with the late phase of pregnancy. A similar rise in leptin level during mid-late pregnancy is also described in two other bat species, M. lucifugus (Widmaier et al. 1997) and Eptesicus fuscus (Kunz et al. 1999). All mammals thus far investigated, including humans, showed increased serum leptin level during mid- to late-pregnancy (Gavrilova et al. 1997, Masuzaki et al. 1997, Kunz et al. 1999, Zhao et al. 2003, 2004). Contrary to the non-pregnant mammals, elevated leptin levels of pregnancy in mammals are not usually correlated with adiposity (Kronfeld-Schor et al. 2001). In baboons, bats, and humans, the placenta is a major source of circulating leptin level during pregnancy (Hoggard et al. 2000, Kronfeld-Schor et al. 2001). The conservation of hyperleptinemia during pregnancy across mammalian orders implies a fundamental role of leptin either in the maintenance of pregnancy or preparation for lactation (Kronfeld-Schor et al. 2000).

The annual variation of serum leptin concentrations shown in the present study also reflected the relationship with the circulating insulin levels in S. heathi (Fig. 2). A low r value obtained in a correlation study may be because more than a single population of bat was compared in the study (Fig. 3). The highest circulating leptin level coincides with the peak circulating insulin level in December in S. heathi. This provides circumstantial evidence supporting the role of insulin in stimulating leptin secretion or vice versa. Many in vitro studies performed with rat and human pancreatic islets, and insulin secreting cell lines provide the evidence for the existence of adipo-insular axis. Leptin shows inhibitory effect, as a negative feedback signal from adipose tissue to the endocrine pancreas, on glucose stimulated insulin secretion (Ookuma et al. 1998, Ceddia et al. 1999, Seufert et al. 1999). Some studies, however, have reported that depending upon the dose of leptin, leptin exposure time, and glucose concentration, insulin secretion from pancreatic β cells is either increased (Shimizu et al. 1997, Tanizawa et al. 1997) or may even remain unaffected (Leclercq-Meyer & Malaisse 1998). Therefore, the ability of leptin to regulate insulin secretion is still a contentious issue due to conflicting results obtained so far. Earlier studies on S. heathi showed the IR and HI during the period of increasing adiposity (Chanda et al. 2003). The period of IR coincides with the period of increased circulating leptin level in S. heathi and further supports the possibility of an interaction between insulin and leptin secretion in this bat. The positive correlation of both leptin and insulin level with the body mass observed in the present study suggests that both leptin and insulin might be linked to the development and maintenance of adiposity in S. heathi as shown in several laboratory rodents (Benoit et al. 2004).

Taken together, our in vivo and in vitro studies on S. heathi suggest that the effects of leptin on ovarian steroidogenesis is mediated by changes in the expression of STAR protein, as recently suggested by Salzmann et al. (2004). The study showed a marked variation in the expression of STAR protein of the ovary of S. heathi during different reproductive stages and showed a close relation with the changes in the circulating leptin level. Increased expression of STAR protein during ovarian recrudescence in October coincides with the low circulating leptin level. Expression of STAR protein decreases during anovulatory period of delayed ovulation and attains its lowest level during December–January, coinciding with the high circulating leptin level. The expression of STAR protein increases again during preovulatory period in February–March when circulating leptin level attains a low level. These observation, thus suggest that in S. heathi, the low level of leptin is clearly stimulatory, whereas the high level is inhibitory to the ovarian STAR protein. This observation is in agreement with a recent study demonstrating biphasic and dose-dependent effects of leptin on STAR expression in porcine granulosa cells (Ruiz-Cortés et al. 2003). The present in vitro study on
the ovary of *S. heathi* also showed that a high dose of leptin impaired expression of StAR protein. Normally, decrease in StAR should result in decrease in both P and A4 synthesis. However, in the present study, decrease in StAR level with leptin treatment associated with enhanced synthesis of both A4 and P by the ovary of *S. heathi*. There may be several possible reasons for this discrepancy. The high dose of leptin might have either enhanced the LH-induced luteinization causing increased P synthesis or it might have suppressed aromatase enzyme activity causing precursors (A4 and P) build up. It is also possible that the effect of leptin on A4 and P may be mediated through StAR independent pathway. It has recently been demonstrated that the inhibition of StAR expression results in a dramatic decline in steroid biosynthesis, although some 10–15% of steroid synthesis continued via StAR independent mechanism (Manna *et al.* 2001, Stocco *et al.* 2005). Since, leptin enhanced both the ovarian expression of insulin receptor protein as well as A4 synthesis simultaneously, *in vitro*, it may be hypothesized that the leptin induced increase in A4 might be through insulin mediated HA in *S. heathi* during the period of delayed ovulation.

Interestingly, the dose of leptin (100 ng/ml), which impaired ovarian expression of StAR protein *in vitro*, had caused up-regulation of expression of insulin receptor protein and A4 synthesis. This *in vitro* study was confirmed by our *in vivo* finding showing markedly increased expression of insulin receptor protein coinciding with the period of high circulating leptin level in December–January in *S. heathi*. That leptin directly modulates insulin receptor in the ovary has so far been not demonstrated. Though Swain *et al.* (2004) have demonstrated in a single cell-culture, unlike the whole ovary-culture in present study, that leptin enhances insulin-stimulated follicular progesterone, testosterone and estradiol synthesis in a dose-dependent manner. Simultaneous increase in expression of insulin receptor protein and androgen synthesis in response to leptin treatment in the same *in vitro* study, thus, suggests a new insight into mechanisms for insulin-mediated androgenicity in *S. heathi*. Our earlier study demonstrated increased insulin-mediated androgen synthesis in *S. heathi* during the anovulatory period of delayed ovulation correspond with the leptin effects observed in the present study (Doval & Krishna 1998). Therefore, one could suggest that both insulin and leptin are involved in HA induced anovulation in *S. heathi* during the period of delayed ovulation. A sharp decline in the ovarian insulin receptor protein *in vivo* during February coincides with the significant decline in circulating leptin level in *S. heathi* and may be responsible for reactivation of folliculogenesis and, subsequently, ovulation in early March in this species.

The effects of pharmacological (1 µg/ml) and high (100 ng/ml) dose of leptin on ovary of *S. heathi* have produced some conflicting results and, thus, have prevented the development of a consistent view of the effect of leptin on ovarian steroidogenesis in the present study. Most of the earlier studies showed that leptin inhibits some combination of gonadotropin and growth factor stimulation of steroidogenesis and, more specifically, estrogen synthesis (Greisen *et al.* 2000). Spicer and associates (Spicer & Francisco 1998, Spicer *et al.* 2000) first demonstrated that leptin impairs insulin or insulin-like growth factor-I in combination with follicle-stimulating hormone stimulation of progesterone and estradiol accumulation in cultured bovine cells. However, other investigators (Kitawaki *et al.* 1999) demonstrated that leptin at 1 ng/ml increased P450 aromatase expression and E2 accumulation in human cells with no effect on progesterone. Spicer & Francisco (1998) further showed an inhibitory effect of leptin on insulin-mediated secretion of A4 from cultured bovine thecal cells. In the present *in vitro* study, however, both high and pharmacological doses of leptin enhanced the stimulatory effect of LH on A4 and progesterone production. Moreover, only the pharmacological dose of leptin suppresses estradiol production by the ovary of *S. heathi* during the period of delayed ovulation. Therefore, it might be presumed that leptin, at pharmacological dose, enhances the LH-induced luteinization of granulosa cells. This finding is further supported by the observations that leptin receptor expression in porcine granulosa cells increases with luteinization both *in vitro* and *in vivo* (Ruiz-Cortés *et al.* 2000) and leptin binding increases with time in the culture of bovine granulosa cells (Spicer & Francisco 1997). Thus, the effect of leptin on A4, P and E2 synthesis in the ovary of *S. heathi* might be mediated either through different pathways or at a pharmacological dose partially through enhancing the LH-induced luteinization of granulosa cells. Similarly, high dose of leptin could enhance insulin receptor protein, whereas pharmacological dose suppressed it. Both high and pharmacological doses of leptin suppressed the StAR protein expression *in vitro* in the ovary of *S. heathi*.

Bats exhibit a variety of reproductive delays, such as delayed ovulation, coinciding with the winter adiposity (dormancy; Krishna 1999). *S. heathi* also showed an increased leptin level during winter dormancy. The results of the present study showed that the increased leptin level during winter dormancy may be responsible for suppressed ovarian activity in *S. heathi* leading to delayed ovulation. The findings of present study, thus may explain why the bats exhibit reproductive delays corresponding with the period of fat deposition during winter dormancy. Increased leptin level suppresses ovulation, had earlier been demonstrated in rat in, which leptin treatment led to a significant reduction in ovulation rates (Duggal *et al.* 2000). Ovulation in *S. heathi* occurs in late February/early March when fat reserves are completely mobilized and corresponding
decline in leptin during this period may be important for the fresh recruitment and maturation of preovulatory follicles (Krishna & Abhilasha 2000). Leptin directly affects the ovary is supported by studies showing the presence of leptin receptor and its mRNA in the human, mouse, rat, and pig ovaries (Karlsson et al. 1997, Ruiz-Cortés et al. 2000, Kikuchi et al. 2001). How does leptin directly affect ovarian activity is however not clearly demonstrated? It has been suggested that high level of leptin decreases ovarian responsiveness to gonadotropin (Agarwal et al. 1999). This may explain why human chorionic gonadotropin (hCG)/pregnant mares serum gonadotrophin treatment failed to induce ovulation in S. heathi during this period (Singh & Krishna 1992). The present study however failed to notice any marked decrease in expression of LH receptor protein during the period of increased circulating leptin level. This study, thus, suggests an important relationship between leptin and insulin, fat reserves and delayed ovulation in S. heathi. The role of some other important factors, such as melatonin, thyroid hormone, and prolactin in seasonal adiposity and anovulation in S. heathi during the period of delayed ovulation requires further investigations.

In brief, our results suggest an association between increased circulating leptin and insulin levels and the seasonal accumulation of adipose tissue before winter dormancy in S. heathi. Circulating leptin levels attain second peak during late pregnancy indicating its role in embryonic development. Increased circulating leptin level also coincides with the anovulatory period of delayed ovulation in S. heathi. The increased leptin level during the period of delayed ovulation impairs ovarian activity by suppressing expression of STAR protein, which in turn results in decrease in E2 synthesis. Simultaneously, the increased leptin level during this period enhances ovarian expression of insulin receptor protein and A4 production. The results of the present study, thus, suggest that high circulating leptin level causes anovulation in S. heathi during the period of winter dormancy by STAR mediated suppression of estradiol synthesis and by insulin receptor mediated hyperandrogenism. These findings suggest that obese women with increased leptin and insulin levels may be more susceptible to ovulatory disorder.

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