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Hyperleptinemia in Pregnant Bats Is Characterized by Increased Placental Leptin Secretion In Vitro

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Hyperleptinemia is a common feature of pregnancy in mammals. The source of increased plasma leptin is uncertain. We examined leptin secretory rates in vitro to test the hypothesis that leptin secretion is upregulated during pregnancy. Two species of insectivorous bats were examined, *Myotis lucifugus* and *Eptesicus fuscus*, because of their unique reproductive cycle. Body mass and plasma leptin significantly increased with gestation and decreased during lactation. Adiposity increased in midgestation, then decreased in late gestation and lactation and was not significantly correlated with plasma leptin in pregnant or early lactating individuals. Leptin secretion in vitro per gram of adipose tissue tended to increase with gestation but was not significantly correlated with plasma leptin in the same individuals. Leptin secretion from placentae, however, increased with gestation and was significantly correlated with plasma leptin from the same individuals. In suckling pups, plasma leptin was high shortly after birth, then decreased to low levels that were not correlated with adiposity thereafter. We conclude that in bats, the placenta is a major source of circulating leptin during pregnancy, and that adiposity and plasma leptin levels are decoupled during three different periods of intense metabolic demand (pregnancy, early lactation, and neonatal growth).

Key Words: Placenta; leptin; bats; lactation; adipose tissue; neonates.

Introduction

Leptin is produced and secreted by adipose cells and acts as a feedback controller of energy balance *(1)*. In addition, animals in which leptin or a functional response to leptin is absent are infertile *(2)*, suggesting that leptin is a necessary component of the hormonal control mechanisms of reproduction. Elevated circulating levels of leptin occur during pregnancy in rats *(3–5)*, mice *(6–8)*, bats *(9,10)*, baboons *(11)*, and humans *(12–18)*. Moreover, the leptin receptor is expressed in uterine *(4)* and placental *(19)* tissue during pregnancy. These observations suggest that leptin may play a role in the maintenance of pregnancy, fetal growth and development, or preparation for lactation.

The source of elevated plasma leptin during pregnancy is unknown, but plasma leptin and adiposity are not always tightly correlated in pregnant females *(4,9,10,13)*, unlike in nonpregnant females in which a positive correlation has been observed *(20)*. This relationship has raised the possibility that leptin may be secreted during pregnancy from nonadipose sites. This hypothesis is consistent with observations that the primate placenta (including human) and cell lines derived from human choriocarcinomas express leptin mRNA at comparable or greater levels than adipose tissue and produce and secrete leptin protein *(11,16,21–25)*. In rodents, however, conflicting results have been reported regarding production of leptin by the placenta *(3, 5–7)*, although we have demonstrated that the mouse placenta does not constitutively secrete leptin protein *(8)*. By contrast, leptin mRNA in adipose tissue *(7)* and the rate of leptin secretion from adipose tissue in vitro *(8)* increase during pregnancy in mice, suggesting that adipose tissue may be the primary source of plasma leptin during pregnancy in rodents. Thus, the regulation of plasma leptin during pregnancy appears to differ among mammalian orders.

The purpose of the present study was to evaluate the source of leptin during pregnancy in bats. Bats were studied for several reasons. First, members of the order Chiroptera comprise roughly 25% of all known mammalian species and are important economically and ecologically as pollinators, seed dispersers, and predators of insects *(26)*. Second, bats and primates appear to be as phylogenetically related to each other as are rodents and primates *(27,28)*. Finally, understanding the control of leptin during pregnancy and lactation in diverse species will extend our knowledge on reproduction in mammals and may provide insight on the physiological functions of leptin during these reproductive states.

We evaluated leptin in two species of temperate-zone, hibernating bats: the little brown bat (*Myotis lucifugus*) and the big brown bat (*Eptesicus fuscus*). The little brown bat

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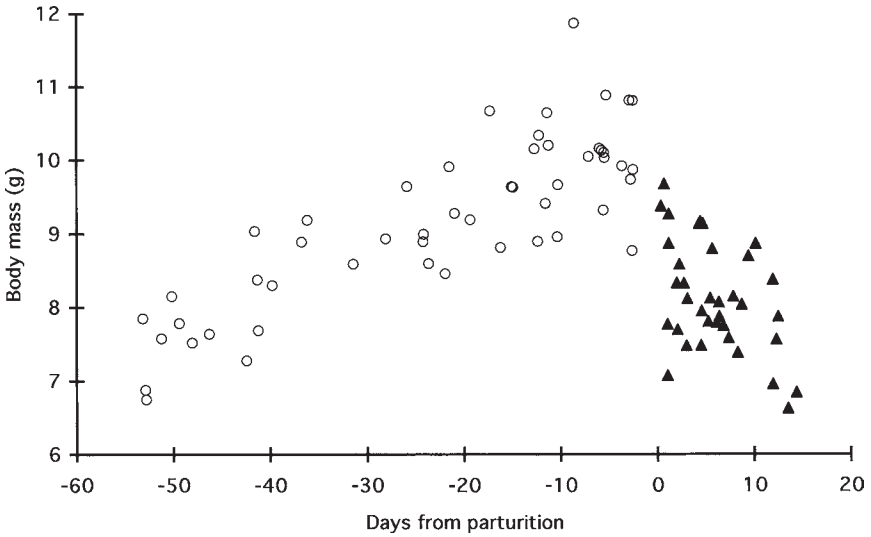

Fig. 1. Body mass of *M. lucifugus* during pregnancy and lactation. Each point represents a single individual. In this and subsequent figures, day of parturition is considered d 0. Negative days indicate gestational stage, and positive days indicate the lactati onal postpartum period. , pregnancy; ▲, lactation. Regression lines are omitted for the sake of clarity. Coefficients of determination (r^2 values) were 0.69 ($p < 0.0001$) for pregnancy and 0.17 ($p < 0.01$) for lactation.

is an insectivore (7–11 g) with a relatively long gestation period (60 d) and produces a single offspring each year. Young bats are functionally altricial at birth but are relatively large, weighing 25–30% of their mother's postpartum mass *(29)*. Young little brown bats begin to forage when they reach about 84% of their mother's postpartum mass and are fully weaned at 26 d. Little brown bats are relatively long-lived and have been known to survive up to 33 yr in the wild *(30,31)*. The big brown bat is larger (18–27 g), survives up to 24 yr, and also has a relatively long gestation period (60 d), producing one to two offspring each year. Young big brown bats are functionally altricial at birth, with individuals weighing 20% of the mother's postpartum mass. The young begin to forage when they reach about 75% of their mother's postpartum body mass. In both species, neonates weigh as much as the entire litter mass predicted for a similar-size terrestrial eutherian *(30,32)*, and they have relatively late sexual maturation (~16 mo for males and 4 mo for females). Thus, in these two species, pregnancy and lactation provide extraordinary metabolic challenges to females. It would be expected, therefore, that leptin synthesis and secretion would be tightly controlled during these periods. We investigated plasma leptin levels in *M. lucifugus* and *E. fuscus* during pregnancy and lactation, to test the hypotheses that leptin is constitutively secreted by the placenta, and that adipose and placental leptin secretion are upregulated as pregnancy proceeds.

Results

As expected, maternal body mass in *Myotis* significantly increased during gestation ($r^2 = 0.69$, $p < 0.0001$) and decreased rapidly following parturition ($r^2 = 0.17$, $p < 0.01$) (Fig. 1). Adiposity significantly decreased prior to parturition ($r^2 = 0.20$, $p < 0.001$) and remained low during lactation (Fig. 2). Maternal plasma leptin levels, by contrast, significantly increased throughout pregnancy ($r^2 = 0.64$, $p < 0.0001$) and significantly decreased within 1 wk following parturition ($r^2 = 0.63$, $p < 0.0001$) (Fig. 3; data for *E. fuscus* shown in Table 1). Adiposity, therefore, was not significantly correlated with plasma leptin levels during pregnancy or early lactation (data not shown). However, a significant positive correlation ($r^2 = 0.62$; $p < 0.01$) between adiposity and plasma leptin was found in lactating females from d 4 of lactation onward (Fig. 4).

Sufficient adipose tissue was not consistently available to examine multiple time points of leptin secretion in each individual. Thus, leptin secretion was first determined in vitro over 2 h in a random sample of bats to verify that constitutive secretion increased with incubation time. In vitro secretion of leptin from adipose tissue increased from 57 ± 12 ng/g of dry fat at 60 min of incubation to 87 ± 19 ng/g of dry fat at 120 min ($p < 0.01$, $n = 16$). Thus, comparisons between females of different gestational stage were made for 120 min. Secretion of leptin per gram of adipose tissue in vitro significantly ($p < 0.002$) increased during pregnancy in *Myotis* (Fig. 5) but not in *Eptesicus* (Table 1). The correlation between adipose leptin secretion in vitro and gestation time in *Myotis*, however, was weak ($r^2 = 0.29$), owing to the highly variable leptin secretory rates near the end of gestation (Fig. 5). In vitro secretion of leptin per gram of adipose tissue was not significantly correlated with plasma leptin in either species (data not shown).

Accumulation of leptin in the culture media from minced placentae increased linearly as a function of time in a random sample of pregnant females (Fig. 6). As with adipose

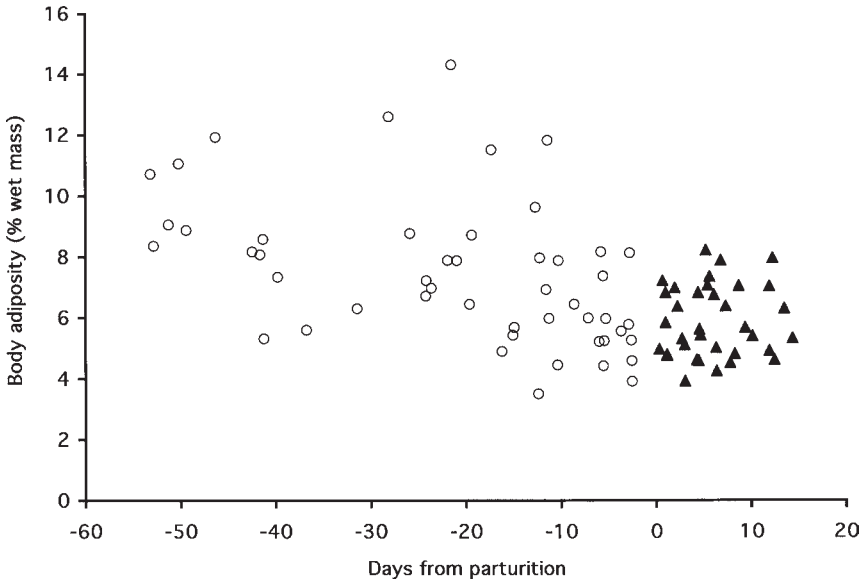

Fig. 2. Body adiposity (%) in *M. lucifugus* during pregnancy and lactation. Each point represents a single individual. , pregnancy; ▲, lactation. Regression lines are omitted for the sake of clarity. The coefficient of determination (r^2) was 0.20 ($p < 0.001$) for pregnancy. There was no significant correlation for lactation.

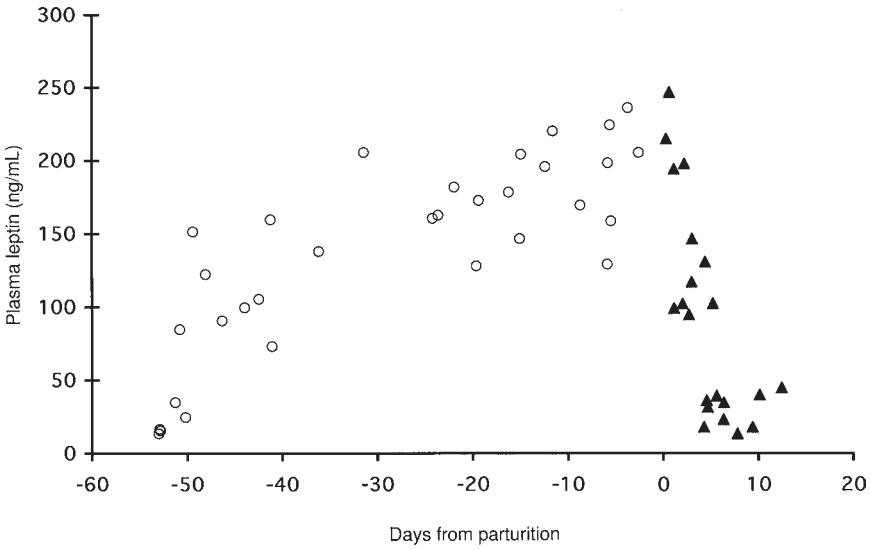

Fig. 3. Plasma leptin levels in *M. lucifugus* during pregnancy and lactation. Each point represents a single individual. These data are from a subset of animals in Fig. 1 for which blood was available for leptin assays. , pregnancy; ▲, lactation. Regression lines are omitted for the sake of clarity. Coefficients of determination (r^2 values) were 0.63 ($p < 0.0001$) for pregnancy and 0.73 ($p < 0.0001$) for lactation.

Table 1
Relationships Among Plasma Leptin, Adipose Leptin Secretion In Vitro, Placental Leptin Secretion In Vitro, and Stage of Pregnancy in *E. fuscus*[a]

Correlation	r^2	p value
Plasma leptin vs stage of pregnancy	0.60	<0.01
Adipose leptin secretion in vitro vs stage of pregnancy	0.08	NS
Placental leptin secretion in vitro vs stage of pregnancy	0.54	<0.01
Plasma leptin vs placental leptin secretion in vitro	0.78	<0.01

[a]NS, not significant; r^2, coefficient of determination; $n = 23$.

tissue, sufficient placental tissue for multiple time points was not consistently available; thus, subsequent comparisons between individuals were made at 120 min. Leptin secretion from minced placentae in vitro significantly increased throughout pregnancy in *Myotis* ($r^2 = 0.75$, $p < 0.01$) (Fig. 7) and *Eptesicus* (Table 1) and was significantly correlated with plasma leptin in both species ($r^2 = 0.48$, $p < 0.01$) (Fig. 8, Table 1). There was no significant correlation between placental mass and rate of constitutive leptin secreted per gram of placental tissue during different stages of gestation (data not shown).

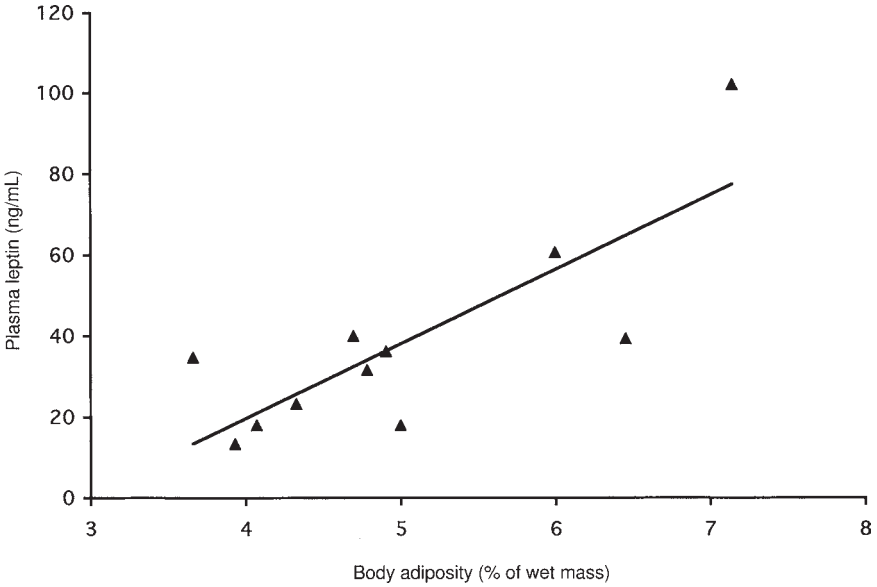

Fig. 4. Correlation between body adiposity and plasma leptin in individual lactating *M. lucifugus* onward from d 4 of lactation ($r^2 = 0.62$, $p < 0.01$). These data are derived from animals represented in Figs. 2 and 3 for which both determinations were available; each point represents a single individual.

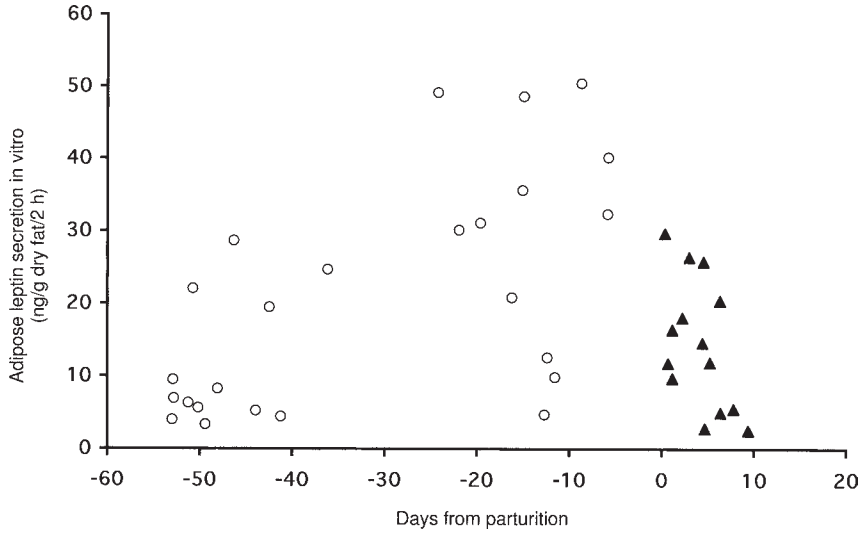

Fig. 5. Leptin secretion in vitro from adipose tissue of pregnant and lactating *M. lucifugus* (from animals in Fig. 3 for which sufficient fat was available for leptin secretion analysis). Each point represents a single individual. , pregnancy; ▲, lactation. The coefficients of determination (r^2) were 0.29 ($p < 0.002$) for pregnancy and 0.27 ($p < 0.056$) for lactation.

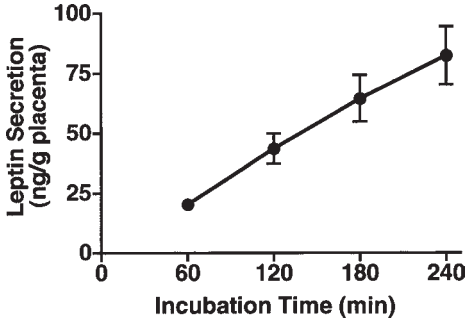

Fig. 6. Secretion of leptin from isolated, minced placentae of *M. lucifugus* over 4 h ($n = 9$, mean ± SEM). Media were collected after each hour, centrifuged to remove debris, and frozen for radioimmunoassay (RIA). Fresh media were added to the tissue incubates for each additional hour.

Plasma leptin levels in suckling pups decreased within 5 to 6 d following birth to low, stable levels (Fig. 9). Adiposity of pups significantly increased with age (Fig. 10) ($r^2 = 0.66$, $p < 0.01$) and was not significantly correlated with plasma leptin (not shown).

Discussion

Recent reports suggest that leptin may have important roles in mammalian reproduction, including onset of puberty, maintenance of fertility, and fetal development (for review see refs. *33–35*). Consistent with this hypothesis, circulating leptin levels increase in pregnant rodents *(3–6,8)*, baboons *(11)*, humans *(12–17)*, and bats (*[9,10]*; this study). How-

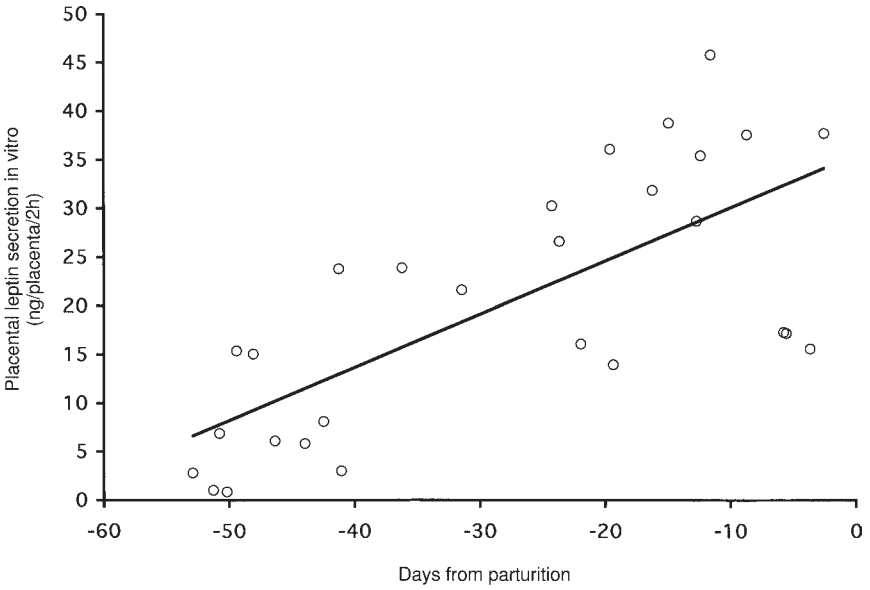

Fig. 7. Leptin secretion in vitro from placentae of little brown bats during different stages of pregnancy ($r^2 = 0.75$, $p < 0.01$). Each point represents a single individual. Data refer to leptin secretion per placenta; when normalized to per-gram placental tissue to account for the increase in placental mass during gestation, there was no significant change. Thus, constitutive leptin secretion depended on placental mass.

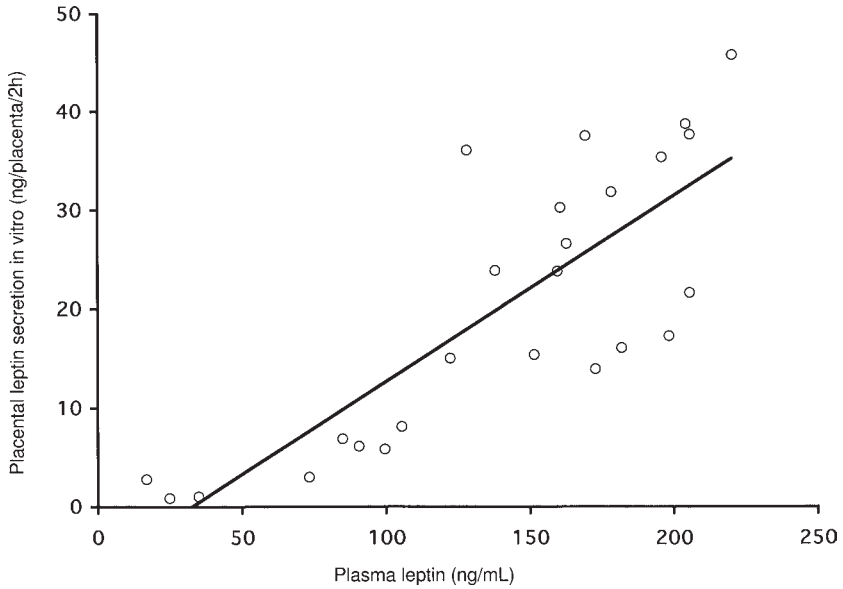

Fig. 8. Correlation between plasma leptin in pregnant *M. lucifugus* and leptin secretion in vitro from placentae of the same individuals ($r^2 = 0.48$, $p < 0.01$). These data are derived from Figs. 3 and 7; each point represents a single individual.

ever, the source of circulating leptin during pregnancy appears to differ among species. Human and baboon placentae express leptin mRNA *(11,21,23)*, but the situation is less clear in rodents, in which leptin mRNA is sometimes *(3,36)*, but not consistently *(5,6)*, observed in placenta. In rodents, it is more likely that leptin expression from adipose tissue is upregulated during pregnancy. This hypothesis is supported by the observation that leptin mRNA increases in adipose tissue during pregnancy in mice, with a time course roughly similar to that of the rise in circulating levels *(5,7)*. In addition, we have recently reported *(8)* that the rate of leptin secretion from adipose tissue in vitro increases in pregnant mice and is highly correlated with circulating leptin from the same individuals. By contrast, we found that leptin in mice is not constitutively secreted by placental tissue *(8)*. Collectively, these results strongly suggest that upregulation of adipose leptin mRNA during pregnancy in mice is accompanied by increased leptin secretion by adipose tissue, and that this continues for a brief period following parturition. Thus, we suggested that any placental contribution to hyperleptinemia is of secondary or no importance in pregnant mice *(8)*.

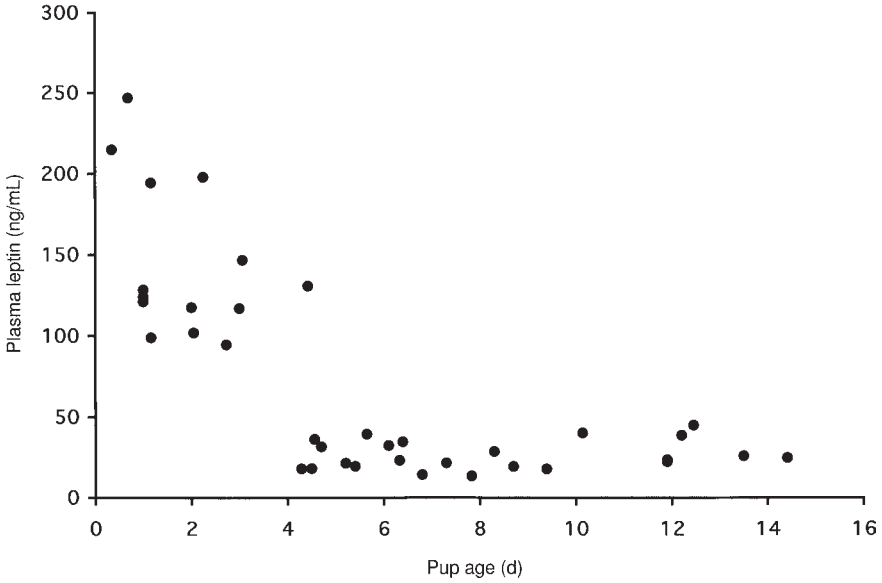

Fig. 9. Plasma leptin levels in suckling *M. lucifugus* pups during early to mid-lactation. Each point represents a single individual.

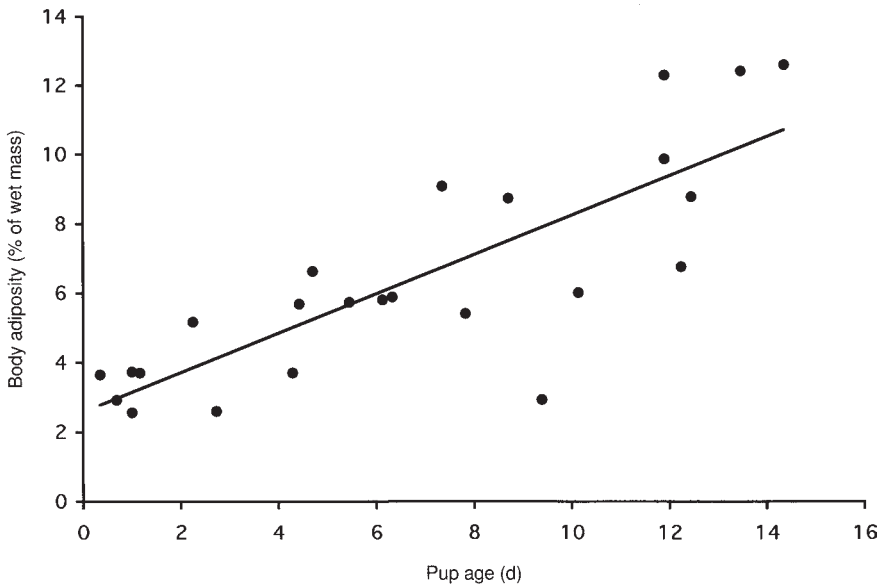

Fig. 10. Body adiposity (%) in suckling *M. lucifugus* pups during early to mid-lactation ($r^2 = 0.66$, $p < 0.01$). Each point represents a single individual.

Here, we report that plasma leptin progressively increases during pregnancy, and decreases within 5 to 6 d following parturition in two species of insectivorous bats, thus confirming and extending our previous results with these two species *(9,10)*. The level of leptin in plasma of pregnant bats reached values 10–20 times greater than those found in adult males (unpublished observations) and in postlactational females *(10)*. Adiposity was highest during early to mid-gestation but decreased prior to parturition and remained low during lactation. The higher percentage of body fat observed during early to mid-pregnancy most likely results from an increase in food consumption at this time *(37)*. Similar patterns were reported for cotton rats *(38)* and humans *(39)*, in which maternal hyperphagia leads to increased maternal fat storage during the first two trimesters. During this period, nutritional needs for fetal growth are minimal *(39)*. The final phase of pregnancy is largely catabolic with respect to the mother, and this persists until parturition. During the third trimester, maternal food intake increases, and fat storage decreases in synchrony with fetal growth *(39,40)*. Fat reserves deposited during pregnancy and early lactation are mobilized during late stages of lactation, when energy demands of the suckling young are highest *(38)*.

Adiposity in female bats was not significantly correlated with plasma leptin levels during pregnancy or early

lactation. The decrease in plasma leptin following parturition and the lack of correlation between adiposity and plasma leptin during pregnancy and early lactation in bats suggests that the usual role of leptin as a signal of fat storage is diminished during these periods. Consistent with this idea was the observation that in vitro secretion of leptin from adipose tissue by pregnant *M. lucifugus* and *E. fuscus* was not significantly correlated with circulating leptin levels from the same individuals during pregnancy, despite a weak but significant trend toward increased secretion rates in late pregnancy. It is possible that the failure to observe a strong relationship between secretion rates in vitro and stage of gestation may be owing to the high variability in secretory rates from adipose tissue collected from females during the final two weeks of gestation. This is a period of metabolic flux that coincides with the mobilization of maternal fat depots and reduction in maternal adiposity, as noted herein. It is possible that during this transient period, leptin secretory rates are less tightly coupled to rapidly changing fat mass, while plasma leptin levels are maintained by the growing placenta. The return of a strong positive relationship between plasma leptin and adiposity onward from d 4 of lactation in lactating mothers, however, suggests that leptin resumes its role as an indicator of adiposity at this time.

By contrast, leptin secretion from placental tissue in both species of bats was strongly and significantly correlated with circulating leptin levels in the same individuals throughout pregnancy. Interestingly, the amount of leptin secretion per placenta was determined entirely by the mass of the placenta. Thus, there was no evidence that the rate of leptin secretion/placental cell was upregulated during later stages of pregnancy in these species. Note, however, that we did not examine the possibility that leptin secretion from placenta is differentially regulated during the course of gestation. Our studies only examined constitutive secretion. Thus, putative circulating regulatory factors present during pregnancy could conceivably further increase placental leptin secretion. It is also recognized that the placenta is a complex tissue with a high metabolic demand, and it is not clear how readily in vitro results can be extrapolated to the in vivo condition. Notwithstanding, the high correlation between leptin secretion per placenta and plasma leptin, and the lack of a similar strong correlation between adipose leptin secretion and plasma leptin, argues strongly for a major placental contribution to the hyperleptinemia during pregnancy in these species.

Thus, it appears that in bats the placenta is a primary but not exclusive source of the progressive increase in circulating leptin during pregnancy. The results are consistent with placental leptin expression reported for human pregnancies. In this respect, bats such as *Myotis* might serve as a more appropriate animal model than laboratory rodents for the study of the physiology of leptin secretion in human pregnancy, although the practicality of the model is limited by the difficulty of maintaining captive colonies.

Plasma leptin in suckling pups decreased dramatically in the first few days after birth. Adiposity of the pups, however, significantly increased with age and was not correlated with plasma leptin levels. A similar decrease in plasma leptin levels from birth to 4 wk of age was observed in human newborns *(41)*. The dissociation between adiposity and circulating levels of leptin in neonates may be a mechanism for stimulating appetite, which is necessary for rapid neonatal growth.

In conclusion, our results suggest that the placenta may be a major contributor to elevated plasma leptin during pregnancy in bats, with upregulation of adipose leptin secretion making a minor additional contribution. Leptin does not appear to function as a signal for adiposity during this time. Its functions during this period in any species are uncertain, but it may be important for regulating growth and development during the fetal and neonatal periods *(33)*, or in preparation for lactation (e.g., by stimulating prolactin production; *[42,43]*). The presence of leptin receptors in the placenta, e.g., suggests the possibility that leptin may serve autocrine or paracrine roles in placental function *(33–35)*. Beginning several days following parturition, leptin appears to resume its function as a signal of adiposity in female bats. The sudden decrease in leptin levels observed in the neonates and in the mother following parturition may be part of the mechanism that contributes to increased appetite in these critical periods of an animal's life history.

Materials and Methods

Collection of Animals

Little brown bats (*M. lucifugus*) and big brown bats (*E. fuscus*) were collected using a harp trap *(44)* from barns located in central Massachusetts and Southern New Hampshire, from May to July, 1998–2000. Bats were trapped 2 to 3 h after dark as they returned from foraging and were immediately transferred to Boston University in simulated roosts *(45)* without additional food. Lactating mother/pup pairs were hand captured from roosts after foraging and pups remained with mothers until the animals were sacrificed. All procedures were approved by the Boston University Institutional Animal Care and Use Committee.

Sample Collections

Approximately 10–12 h after capture (~9:00 AM), bats were weighed and sacrificed by decapitation to obtain plasma for hormone assays. This period of fasting is similar to the amount of time spent roosting without food and water in the wild and also eliminates the possibility of feeding-induced changes in leptin secretion in *Myotis (10)*. Embryos were removed and weighed to establish the stage of pregnancy based on a regression equation of embryo mass vs gestational age in *M. lucifugus* (unpublished data). Stage of lactation was assessed from the age of the pup from each mother–pup pair, based on age-estimation equations previ-

ously published for this species *(46)*. Plasma was obtained from trunk blood and frozen prior to leptin RIA. Subcutaneous fat and placentae, which are hemochorial in bats *(47)*, were immediately dissected on sacrifice.

Incubations and Hormone Assays

Fat and placentae were weighed, minced, and washed to remove residual extracellular fluid and blood cells. Adipose tissue (~50 mg wet mass) from each animal was then aliquoted into glass test tubes in 1 mL of serum-free Krebs buffer supplemented with 40 g/L of bovine serum albumin, and 25 μg/mL of leupeptin and aprotinin. Samples of adipose tissue and placentae (1 minced placenta/tube in 1 mL of buffer) were incubated for 120 min at 37°C in a humidified 95% O_2/5% CO_2 chamber with moderate shaking (~1 cycle/s), as previously described *(8,48)*. Media (infranates) were then collected, dehydrated with a Speed-Vac, and stored at –20°C. Leptin concentrations in plasma and media were determined by RIA using a human leptin RIA kit (Linco) that was previously validated for bat serum leptin *(9,10)*. Dried media samples were first reconstituted to 10% original volume with assay buffer. Blank samples (no tissue) were processed similarly and subtracted from the RIA. At the conclusion of an experiment, adipose tissue from each tube was collected and dried at 60°C for 3 d to obtain dry fat mass in order to normalize secretion data between experiments.

In preliminary experiments, linearity of constitutive leptin secretion from minced adipose and placental tissue was determined. Media were collected and processed for RIA every hour for 2–4 h. The media were replaced with fresh media after each collection.

Fat Extractions

Carcasses (without embryos and placentae) were weighed, minced, and desiccated in a drying oven at 60°C until constant mass was achieved *(49)*. Body fat was extracted from the desiccated subjects using a Soxhlet apparatus with petroleum-ether and ethanol *(1:3)* as the organic solvent *(49)*. Each sample was fluxed with the organic solvent for approx 24 cycles (45 min each). The extracted carcasses were dried to constant mass at 60°C to generate a value for lean dry mass. Fat mass was calculated as the difference between dry mass and lean dry mass. Stomachs were assumed to be void of food at the time of sacrifice since the bats were held in captivity for approx 10–12 h following capture. Consequently, stomach contents were not removed prior to extraction. The dry mass of adipose tissue that was removed for in vitro secretion experiments was added to the calculated fat mass of the extracted carcasses.

Statistical Analysis

Coefficients of determination and statistical significance levels were obtained by subjecting data to a best-fit analysis according to a linear regression model using the PRISM software from GraphPad.

Acknowledgments

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