

ORIGINAL PAPER

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Plasma leptin decreases during lactation in insectivorous bats

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Abstract We previously demonstrated high leptin levels during late pregnancy in little brown bats (*Myotis lucifugus*). We now extend these observations to a second species, the big brown bat (*Eptesicus fuscus*), and also report that leptin increases after the first trimester of pregnancy. Leptin decreased to baseline 1 week following parturition, with a half-time decay of 2 days. During lactation, leptin was significantly correlated with body mass in *E. fuscus*, but not in *M. lucifugus*. No circadian pattern of leptin was observed in *M. lucifugus*. The decrease in post-partum leptin in bats may be partly explained by loss of putative placental leptin. The continued decrease may reflect depletion of body fat during this energy demanding period, at least in *Eptesicus*. Changes in leptin during lactation appeared to be independent of circadian effects and time of sampling. Our study provides additional evidence that leptin increases during pregnancy and declines during lactation in a free-ranging mammal, supporting the hypothesis that leptin plays important but yet undetermined roles in reproduction.

Key words Chiroptera · Energy balance · Lactation · Leptin · Pregnancy

Introduction

Leptin, the product of the *ob* gene first identified in mice (Zhang et al. 1994), suppresses appetite (Halaas et al. 1995) and restores fertility in strains of obese rodents (Ahima et al. 1996; Barash et al. 1996; Chehab et al. 1996). Among mammals that have been studied, the

highest reported circulating levels of plasma leptin are from the little brown bat (*Myotis lucifugus*), where in late pregnancy leptin levels reach 150 ng/ml (Widmaier et al. 1997). Similarly, increases in circulating leptin have been reported in pregnant women (Butte et al. 1997) and rodents (Chien et al. 1997). Leptin levels, however, are not strongly correlated with body fat during pregnancy in either humans (Butte et al. 1997) or *M. lucifugus* (Widmaier et al. 1997); in women the major source of circulating leptin during pregnancy may be of placental origin (Masuzaki et al. 1997b). These observations, together with observations that appetite in mammals is not suppressed during pregnancy, suggest that leptin resistance may exist during pregnancy.

Leptin levels are lower during lactation than in pregnancy both in humans (Butte et al. 1997) and bats (Widmaier et al. 1997). However, the latency, rate, and persistence of the decline in leptin during lactation has not been characterized in the early post-partum period. Reduced leptin levels during lactation, the most energetically demanding period in a mammal's life history, are consistent with hyperphagia observed during this period (Kunz 1974; Anthony and Kunz 1977; Kunz et al. 1996). A more complete evaluation of temporal changes in circulating leptin during pregnancy and lactation may help elucidate the putative roles of this hormone in the maintenance of reproduction, including factors that may contribute to its increase during pregnancy and decrease during lactation.

In the present study we extended our previous observations on *M. lucifugus* to include results from another chiropteran species (*Eptesicus fuscus*) which has a similar life history, and also by evaluating levels of leptin in the first trimester of pregnancy. The big brown bat, *E. fuscus*, is notable for producing two pups in a single annual litter which may approach 40% of the mother's postpartum body mass (Burnett and Kunz 1982). Because the energetic demands of lactation in this and other bat species are relatively high (Kurta et al. 1989; Kurta et al. 1990; Kunz et al. 1996), and as a consequence appetite and energy consumption are greatly

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increased, we expected to observe low circulating levels of leptin shortly after lactation began.

Materials and methods

M. lucifugus (ca. 7–12 g) and *E. fuscus* (ca. 15–26 g) are relatively small insectivorous bats native to North America (Fenton and Barclay 1980; Kurta and Baker 1990). Mating occurs in September to October followed by sperm storage during the hibernating period (October to March), and delayed ovulation and fertilization occur in spring (Buchanan 1987). The gestation period in both species ranges from 50–70 days (Wimsatt 1945; Christian 1956); the variation in gestation length depends on yearly changes in climate, since both species are subject to torpor during pregnancy. In the northeastern United States, parturition typically occurs from mid-June to early July in *Myotis*, and from early to mid-June in *Eptesicus*. Lactation of a single pup in *Myotis* and two pups in *Eptesicus* continues through mid-July. Feeding in both species typically involves two nightly foraging bouts from approximately 2030 hours to 2330 hours and from 0300 hours to 0430 hours, with an intervening night-roosting period (Anthony and Kunz 1977; Anthony et al. 1981; Kurta et al. 1989; Kurta et al. 1990).

Animals were captured either in harp traps or mist nets (Kunz and Kurta 1988), or directly removed from the day roosts at selected maternity colonies in New Hampshire and Massachusetts, as previously described (Anthony and Kunz 1977; Burnett and Kunz 1982). Immediately upon capture, females were individually marked with numbered wing bands (Lambournes), weighed, and bled from a vein in the tail membrane (Kunz and Nagy 1988). For *Myotis*, the age of suckling pups was estimated from the lengths of the forearm and total epiphyseal gaps (Burnett and Kunz 1982; Kunz and Anthony 1982), and the stages of lactation of the mother were determined from these ages (Kunz et al. 1995). For *Eptesicus*, we were able to capture and band several individuals on the day of parturition. Some of these banded bats were later recaptured during lactation, enabling us to determine the precise day of lactation of the mother. All bats were released at the site of capture.

Typically, all female *Myotis* and *Eptesicus* that return each spring to maternity colonies are pregnant, with very rare exceptions (Wimsatt 1945; Christian 1956). Stages of pregnancy (first, second, and third trimester) were estimated from abdominal palpation. Briefly, based on descriptions for a similar bat species (Racey 1974), and our own unpublished work on gestation and fetal development during gestation in *M. lucifugus*, the following criteria were developed for assigning bats in maternity colonies to a given gestational stage. During the first trimester, the fetus cannot be detected by hand. During the second trimester, the fetus can be detected by palpation, but no cartilage or bone has formed. In the third trimester, the fetus can be detected by palpation based on the presence of formed cartilages and a bony skeleton. Assuming an average gestational period of 60 days, each trimester lasts approximately 3 weeks.

To determine whether a diurnal rhythm of circulating leptin exists in bats, post-lactating *M. lucifugus* were captured and placed into simulated wooden roosts (Kunz and Kurta 1988) at the field site and given water but no food. While in these simulated roosts, the bats were exposed to environmental conditions (including the light/dark cycle) comparable to those in their natural roosts. We used post-lactating females to test for possible circadian effects to avoid any hormonal or behavioral cues from attached pups which could potentially serve as zeitgebers. Fasted animals were examined because feeding is associated with increases in leptin levels in bats (Widmaier et al. 1997), and we wished to avoid inducing factors (e.g., insulin) that could obscure an underlying photoperiod-driven rhythm by directly altering leptin expression or secretion. Cessation of lactation was verified by the reduction in size of mammary glands (Kunz et al. 1995). Different random samples of post-lactating females were bled approximately every 2 h for 24 h to test for circadian effects.

Blood was centrifuged immediately after collection, and the plasma portion frozen for later analysis. Leptin was determined in 10 μ l plasma samples from bats using a human leptin radioimmunoassay (RIA) kit from Linco, as previously described (Widmaier et al. 1997). At this plasma dilution, samples fell within the linear range of the standard curve. Bat leptin is recognized primarily by anti-human leptin antisera, and dilutes in parallel with human leptin standards (Widmaier et al. 1997). Recovery of a known quantity of human leptin added to bat plasma is 100% (Widmaier et al. 1997), suggesting that putative plasma binding proteins, if they exist, do not interfere with the RIA. A subset of samples were independently analyzed by the manufacturers of the leptin RIA kit. The results of those assays confirmed that bat leptin is most readily detected by the human leptin assay, although it is also detectable by the antisera employed in the universal leptin kit (Linco Research, personal communication). The inter-assay coefficient of variation was approximately 13% at a mean of 18.2 ng/ml ($n = 9$). Data were analyzed using one-way ANOVA and Duncan's post-hoc test, or by unpaired *t*-tests where appropriate. Curve fitting was accomplished by non-linear or linear regression analysis using the Prism software package from GraphPad. All protocols were approved by the Boston University Animal Care and Use Committee.

Results

Leptin levels in *E. fuscus* recorded during different stages of reproduction are shown in Fig. 1. Leptin was highest during pregnancy and decreased significantly (by more than 50%) during lactation. All pregnant bats were in the third trimester of pregnancy, except for one individual who was in the second trimester.

In a previous study we found high leptin levels (75–150 ng/ml) in pregnant *M. lucifugus* during the second and third trimesters (Widmaier et al. 1997), but were unable to obtain first trimester animals at that time. We now report that leptin is low during the first trimester of pregnancy in *Myotis* (22 ± 1 ng/ml, $n = 16$; not shown).

The data for lactating bats summarized in Fig. 1 include all females for whom lactation was confirmed by manual expression of milk. For a subset of these individuals, we determined the precise day of lactation based on individuals that were previously captured and

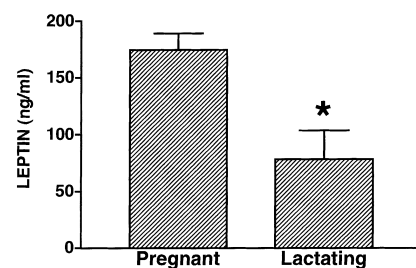


Fig. 1 Circulating leptin profiles in pregnant ($n = 7$) and lactating ($n = 21$) *Eptesicus fuscus* upon return from foraging. Animals were captured, bled, examined for reproductive condition, and released. Leptin was determined in plasma using the human leptin kit, previously validated for chiropteran plasma (Widmaier et al. 1997). Results are expressed as means \pm SE. An asterisk (*) indicates significance at the $P < 0.01$ level in a comparison of pregnant vs lactating females

marked on the day of parturition. In addition, mother-pup pairs of *M. lucifugus* were captured, bled (mothers only), individually marked (as described above), weighed, measured (pups only), and released. The data in Fig. 2 and 3 show that leptin levels decreased following parturition, with a half-time decay of approximately 2–3 days in both species. Leptin levels remained low and stable in both species after 5–10 days of lactation.

Body mass, an index of fat mass in *M. lucifugus* (Kunz et al. 1998) was not significantly correlated with the stage of lactation in *M. lucifugus* but was correlated in *E. fuscus* (Fig. 2, 3). During lactation, body mass and leptin were weakly but significantly correlated in *E. fuscus* ($r^2 = 0.45$, $P < 0.01$), and were not correlated in *M. lucifugus* (not shown).

Because the data presented in Figs. 2 and 3 were not all obtained at the same time of day, and because leptin has been reported to vary diurnally in other species, we sampled bats over a 24-h period to profile circulating leptin levels in *M. lucifugus*. A sufficient sample was not available for the same experiment in *E. fuscus*. Food was withheld to eliminate the possible bias associated with feeding-induced changes in leptin. The results of a pilot study to determine the effect, if any, of non-specific handling/captivity stress on bats placed into simulated roosts did not reveal a significant effect of time in cap-

tivity on circulating leptin, although at approximately 30 min after placement in the roosts there was a transient tendency for some animals to demonstrate higher leptin levels (not shown). However, no significant diurnal change in leptin was observed over 24 h in fasting, post-lactational *M. lucifugus* (Fig. 4). This indicates that time of day in *M. lucifugus* does not influence leptin measurements in fasted animals, and that an endogenous rhythm of leptin may not exist at this time of year in this species.

Discussion

We have shown that circulating leptin in *E. fuscus* is highest during pregnancy and lowest during lactation, observations which are consistent with our previous findings in *M. lucifugus* (Widmaier et al. 1997). Indeed, these two species have the highest reported leptin levels among the mammalian species sampled to date, and in fact higher than has been reported in ventromedial hypothalamic-lesioned rats (Satoh et al. 1997) and in humans with Cushing's disease (Cizza et al. 1997; Masuzaki et al. 1997a). Recently, very high circulating levels of leptin were reported in pregnant women (Butte et al. 1997; Masuzaki et al. 1997b), although the absolute values in women were considerably lower than in

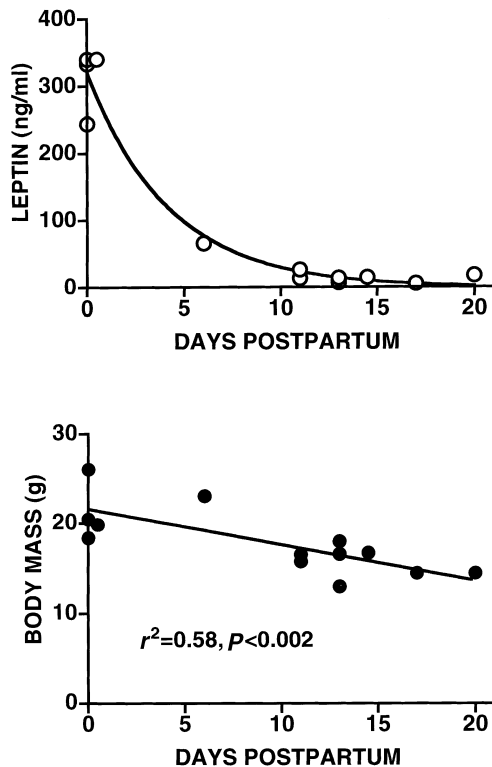


Fig. 2 Leptin levels and body mass in lactating *E. fuscus* whose stage of lactation was determined in animals that were previously captured and banded on the day of parturition (see Materials and methods). Each symbol represents a single animal. Body mass in this figure and in Fig. 3 refers to the mass of the lactating mother only

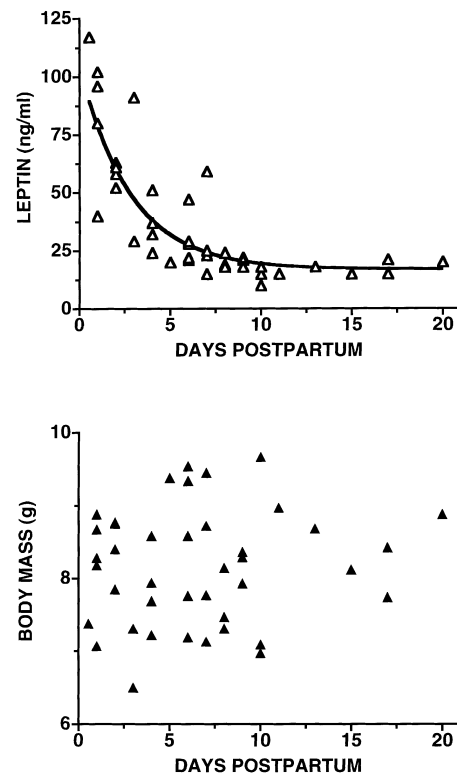


Fig. 3 Leptin levels and body mass in lactating *Myotis lucifugus* whose stage of lactation was estimated from the age of an attached pup (see Materials and methods). Each symbol represents an individual animal

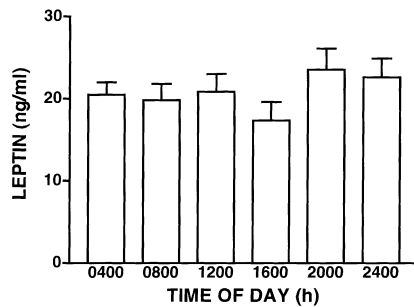


Fig. 4 Twenty-four-hour profile of plasma leptin levels in *M. lucifugus*. Post-lactating animals were captured prior to leaving the roost for nightly foraging, and housed in a simulated roost for up to 24 h without food, but with water. Individual bats were bled once at roughly 2-h intervals throughout the 24-h period. No bat was bled more than one time. Results from pooled data from 4-h intervals are shown for the sake of simplicity; thus, for example, the data shown at 0400 hours represent all bats sacrificed between midnight and 0400 hours. Each bar includes values from 6–10 individuals. There was no significant effect of time of day on plasma leptin whether the data were analyzed by 2-h, 4-h, or 12-h intervals, or when individual data were plotted as a scattergram over the 24-h period

the two species of bats examined in the present study. In women, the placenta contains immunoreactive leptin and leptin mRNA, and presumably is a source of elevated plasma levels observed during pregnancy (Bi et al. 1997; Masuzaki et al. 1997b). Although we have yet to characterize leptin in the bat placenta, the progressive rise in circulating leptin to values ten times greater than baseline is consistent with a placental source. Also consistent with a non-adipose source of leptin during pregnancy in bats is the very weak correlation between body adiposity and plasma leptin during pregnancy (Butte et al. 1997; Widmaier et al. 1997).

The physiological significance of elevated leptin during pregnancy is unknown for any species. In this report, we show that leptin levels are relatively low during the first trimester of pregnancy in *M. lucifugus*, whereas previously we have documented very high and rising levels in the second and third trimesters (Widmaier et al. 1997). Thus, the pattern of leptin in plasma during pregnancy in bats parallels that of other hormones, such as progesterone, estradiol, glucocorticoids, and prolactin, with important roles in maintenance of pregnancy and preparation for lactation. Of these, at least two (estradiol and glucocorticoids) are known to stimulate leptin synthesis in rodents and humans (De Vos et al. 1995; Larsson and Ahren 1996; Masuzaki et al. 1997a; Shimizu et al. 1997).

Whether leptin plays a role in intra-placental communication, or a more systemic role during pregnancy is unknown. However, leptin receptor messenger RNA is expressed in the uterus of rats (Butte et al. 1997; Zamorano et al. 1997), and increases during pregnancy, suggesting the possibility that leptin acts within the uterus to modulate energy balance or otherwise maintain uterine tissue (Chien et al. 1997). At concentrations approximately twice those achieved in plasma in preg-

nant bats, leptin stimulates prolactin production in the anterior pituitary of the rat in vitro (Yu et al. 1997), and thus may be important in the development of mammary glands during late pregnancy. In addition, leptin has been demonstrated to suppress steroidogenesis from cultured bovine ovarian cells (Spicer and Francisco 1997), and thus at high concentrations leptin could conceivably act to suppress ovarian activity during pregnancy.

Plasma leptin declined after birth in *M. lucifugus* and *E. fuscus*. Following an initial rapid decline to levels approximately 50% of pre-partum levels, leptin gradually declined to a stable, low baseline level by 5–10 days post-partum. The initial drop to 50% of pre-partum values took approximately 2 days. The initial rapid drop in leptin may in part reflect loss of putative placental leptin. However, the half-life of circulating leptin in other mammals has been reported to be less than 30 min (Klein et al. 1996). Thus, it is possible that placental leptin alone does not account for the high levels observed during late pregnancy, unless the rate of leptin clearance is dramatically decreased after birth in bats. Consistent with this is the recent demonstration that leptin messenger RNA levels increase in adipocyte tissue during pregnancy in rats and mice (Kawai et al. 1997; Tomimatsu et al. 1997), suggesting that leptin expression is upregulated in fat cells during pregnancy. It is currently unknown, however, if this increased expression is associated with increased secretion of leptin.

The second, more gradual decline in leptin may reflect diminished body fat during early stages of lactation (Burnett and Kunz 1982). Although body adiposity was not determined in this study, body mass was obtained, which is a good predictor of body fat in *M. lucifugus* (Kunz et al. 1998). The steady decline in body mass and leptin in *E. fuscus* is consistent with a post-partum decline in adipose tissue. However, this was not the case for *M. lucifugus*, suggesting that factors other than total body fat are significant during lactation in the regulation of leptin secretion. However, this conclusion must remain tentative until actual determinations of body fat content are made in lactating animals.

The maintenance of lower leptin levels during lactation is consistent with the ingestion of large quantities of food during lactation. Lactating *M. lucifugus* may consume upwards to 100% of their body mass in insects nightly at peak lactation (Kurta et al. 1989), and similar levels are expected for *E. fuscus* (Kurta et al. 1990).

One limitation of the present study was that blood could not always be collected from bats at the same time of day. Because leptin is reported to vary diurnally in rodents (Ahima et al. 1996) and humans (Sinha et al. 1996), this raised the possibility that our results may have been biased by a circadian effect. Thus, we captured animals and placed them in simulated roosts at our field sites to prohibit them from departing at dusk, but otherwise they were exposed to the same ambient conditions of their natural roost. This procedure was comparable to the natural behavior of *M. lucifugus* and

E. fuscus on evenings when adverse weather conditions prevent them from leaving the roost to feed (e.g., Kurta 1986). When animals were randomly selected and bled at roughly 2-h intervals, no evidence for a diurnal rhythm of leptin was observed. This interesting difference between bats and other mammals may reflect the particular sleep-wake (activity) cycle of bats. During lactation, both species of bats are generally active during two discrete foraging periods each night with an intervening 2–5 h night roosting period between foraging bouts (Anthony and Kunz 1977; Anthony et al. 1981). By contrast, free-ranging rodents may consume as much as 80% of their food and water intake in a single, prolonged nightly foraging bout (Saint Girons 1966). Alternatively, the amplitude of a putative leptin rhythm in bats may be obscured during fasting. The latter possibility would be consistent with the situation in rats, in which a diurnal rhythm in leptin was observed only in ad-libitum fed animals (Saladin et al. 1995).

Collectively, rodents and bats constitute roughly 75% of all known mammalian species. We conclude that high circulating levels of leptin may be a widespread phenomenon in pregnant mammals, and that its physiological role need not be necessarily related to the regulation of energy balance. In addition, the observed decrease in leptin after parturition in bats may be too slow to be accounted for simply by the loss of (putative) placental leptin. Recently, leptin has been reported in human milk (Casabiell et al. 1997), raising the possibility that clearance of leptin from the plasma of lactating females may be partly due to sequestration from maternal blood into milk, which in turn is consumed by the suckling juvenile. In view of this hypothesis, it would be of interest to determine the clearance rates of radiolabeled leptin during the different stages of pregnancy and lactation, particularly since a leptin-binding protein has been demonstrated to rise in plasma during pregnancy in rodents (Gavrilova et al. 1997).

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