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Leptin, Corticotropin-Releasing Hormone (CRH), and Neuropeptide Y (NPY) in Free-Ranging Pregnant Bats

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Leptin, the product of the obese gene first identified in mice, restores fertility in obese mice, and accelerates puberty in mice. We hypothesized that leptin's putative role in reproduction may extend to pregnancy and lactation. Leptin levels were determined in *Myotis lucifugus*, the little brown bat, a free-ranging mammal with a seasonal breeding cycle. The present study shows that plasma levels of leptin progressively rise during pregnancy, supporting a potential role for leptin in the maintenancy of pregnancy. In contrast, leptin was significantly lower during lactation, a time when most mammals, including bats, demonstrate reduced fertility. In addition to its possible roles in reproduction, leptin appears important in regulation of energy balance. *M. lucifugus* spontaneously fasts for up to 16 h each day during the active season, which allowed us to test the hypothesis that acute fasting was associated with decreased leptin. Leptin was significantly lower in fasted (lactating) bats, compared to those that recently returned from nightly foraging. Although postprandial lactating bats had a significantly higher fat index than fasted bats, plasma leptin and body fat were not significantly correlated, and were only weakly correlated ($r^2 = 0.26$) when both pregnant and lactating females were included in the analysis. Similar changes during pregnancy, lactation, and the daily feeding cycle were observed in the hypothalamic neuropeptide, corticotropin-releasing hormone (CRH), which is believed to play an important role in energy balance and reproduction. By contrast, neuropeptide Y (NPY) increased during pregnancy but did not change during fasting. These results suggest that leptin's putative role in reproduction may extend to pregnancy and lactation, and that spontaneous, acute fasting results in decreased circulating levels of leptin in *M. lucifugus*.

Key Words: Leptin; pregnancy; bats; feeding; fasting; hypothalamus.

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Introduction

Energy consumption and expenditure in mammals are under the coordinated regulation of peptides in the central nervous system and circulating hormones. Neuropeptide (NPY) induces hyperphagia, decreases thermogenesis *(1–3)*, and is expressed at higher than normal levels in genetically obese rodents *(4)*, but its exact role in energy balance is still uncertain. For instance, normal (nonobese) mice deficient in NPY do not show the predicted aberrations in body mass and feeding *(5)*. On the other hand, when obese (leptin-deficient) mice are made deficient in NPY, body mass and adiposity are partly normalized compared to obese mice with NPY *(6)*. Unlike NPY, corticotropin-releasing hormone (CRH) is considered to be anorexogenic *(7,8)*.

Systemic hormones also regulate body mass, adiposity, and metabolic rate. Of these, the product of the obese gene, leptin, may be of special importance, by acting within the hypothalamus to inhibit the synthesis and secretion of NPY *(9–13)*. Moreover, enforced starvation of laboratory mice is associated with low leptin levels, which may be partly responsible for starvation-induced changes in glucocorticoids, thyroid hormone, and fertility *(9)*. Genetically obese rodents, which lack or are unresponsive to leptin *(14,15)*, exhibit some of the same characteristics as normal, starved rodents, lending further support to the leptin hypothesis.

Most research into the physiological roles of leptin and other factors in the control of feeding has been conducted in artificial (laboratory) settings, often on strains of rodents with mutations in the obese gene or its homolog, or in the leptin receptor. Confirmation of results obtained with such models using free-ranging animals, which are allowed to feed in their natural habitats, should provide important validation of the hypothesis that leptin, NPY, and CRH are major components of the body's adjustment to changes in the postprandial/fasted states. In the present study, we have characterized the circulating and hypothalamic levels of these peptides during two stages of intense energetic demand, pregnancy and lactation, in a free-ranging mammal.

Results

CRH, NPY, and leptin assays were first validated for *Myotis lucifugus*. In each case, the slope of the regression

Table 1
Parallelism of *Myotis lucifugus* Peptides with Standard Curves[a]

	r^2	Slope
NPY	0.99 ± 0.01	1.03 ± 0.06
CRH	0.99 ± 0.01	0.92 ± 0.05
Leptin	0.99 ± 0.01	0.80 ± 0.03

[a]Percent binding for each standard was highly correlated (r^2) with percent binding for four dilutions of *Myotis* plasma or hypothalamic extracts. Values are mean and SE of 5–6 hypothalamic extracts or mean/range of two different plasma pools from five bats each (leptin).

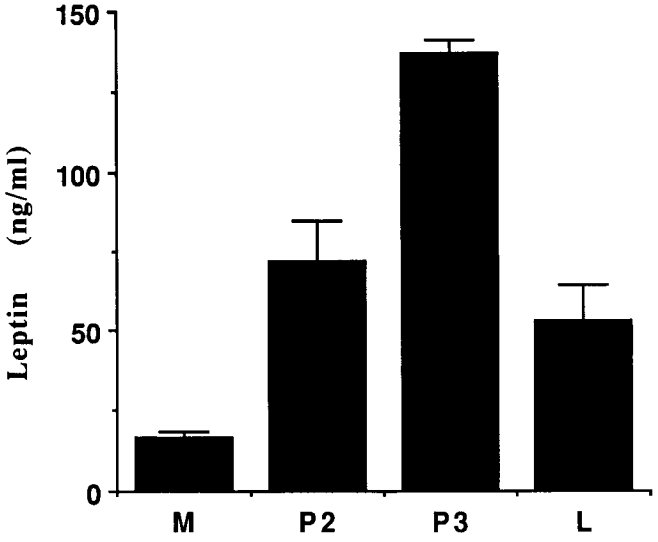

Fig. 1. irLeptin levels in bats. Values are mean and range of two males (M), and the mean and SE of 5–15 females. P2 and P3, second and third trimester of pregnancy (n = 5 and 7, respectively); L, lactating (n = 15). All bats were postprandial, and captured at random over a 40-d span (June–July, 1996).

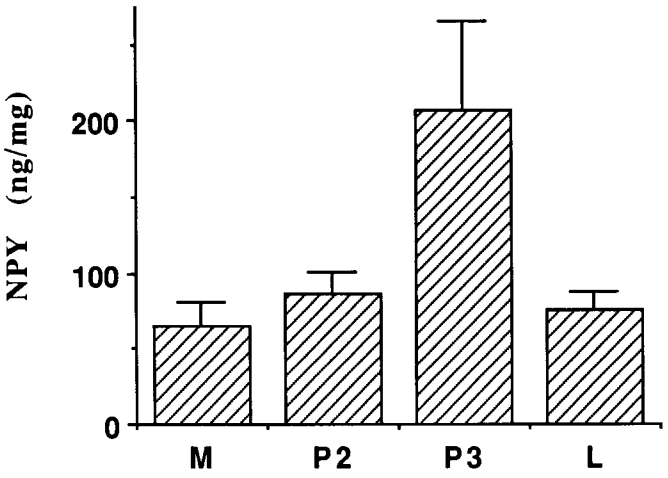

Fig. 2. NPY content of hypothalami of male (M) and female bats of different reproductive condition. Data are expressed relative to mg hypothalamic protein. *See* Fig. 1 for details.

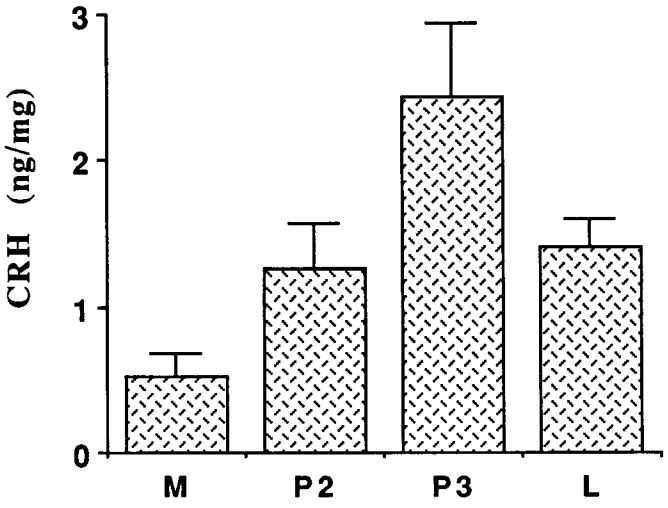

Fig. 3. CRH content of hypothalami of male (M) and female bats. Data are relative to mg hypothalamic protein. *See* Fig. 1 for details.

line ([B/Bo standard] vs [B/Bo extract or serum], where B/Bo is percent binding) was close to 1.0, indicating a direct correlation (Table 1). Thus, semipurified extracts and serum displaced labeled tracer from its respective antibody in parallel with authentic standards. Since the leptin assay is performed on unextracted serum, we further validated this assay by measuring the recovery of human leptin added to a pool of *Myotis* serum. Recovery of leptin from "spiked" serum was ~100% (serum pool, only: 18.6 ± 0.4; human leptin, only: 12.1 ± 0.6; human leptin added to serum pool, 30.6 ± 0.7; all values ng/mL, n = 4).

irLeptin was higher in females than in males, although the number of males captured in the maternity colony was low (Fig. 1). Leptin levels were significantly higher in the third trimester of pregnancy and declined during lactation (Fig. 1). In addition, four females that were "nonreproductive" (neither pregnant nor lactating) were examined. In those, all of whom were fasted, irleptin was 20.2 ± 2.7 ng/mL (not shown). Similar patterns of significant changes in hypothalamic NPY and CRH were observed during pregnancy and lactation (Figs. 2 and 3).

Cortisol levels in fed bats were 121 ± 17, 167 ± 18, and 107 ± 11 ng/mL, in second trimester, third trimester, and lactating bats, respectively (not shown). Cortisol was significantly greater ($p < 0.01$) in P3 bats than during lactation.

We were able to capture lactating bats at both the pre- and postfeeding times of day, from late June to mid-July. Although leptin, NPY, and CRH all showed a trend toward lower levels in fasted bats, only in the cases of leptin and CRH were the changes statistically significant (Fig. 4). In some cases, we were able to capture fasted bats during the second trimester of pregnancy, as well as fasted "nonreproductive" animals. Even when the results were pooled across reproductive condition, CRH and leptin, but not NPY, were significantly lower ($p < 0.01$) during fasting than after feed-

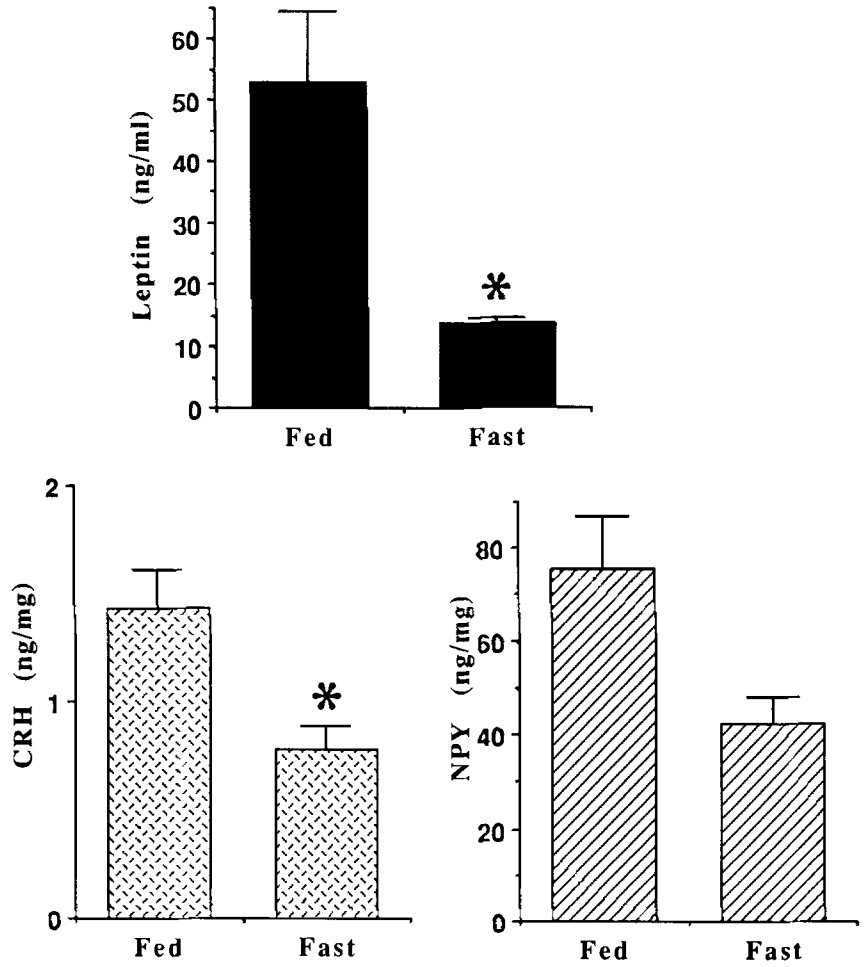

Fig. 4. Circulating leptin and hypothalamic NPY and CRH content in lactating bats before (fast) and after (fed) the first nightly feeding. For each hormone, $n = 5$ for fasted animals; data for lactating/fed values are reproduced from Figs. 1–3 for comparison. *At least $p < 0.05$ vs fed group.

Table 2
Body Fat Index
for Pregnant and Lactating *M. lucifugus*[a]

Condition	Fat index
Pregnant (fasted)	0.42 ± 0.07 (4)
Pregnant (postprandial)	0.40 ± 0.02 (12)
Lactating (fasted)	0.22 ± 0.02[b] (5)
Lactating (postprandial)	0.32 ± 0.01[b] (13)

[a]Values represent mean and SEM of (*n*) animals. Data from pregnant bats in second and third trimester were pooled. Two carcasses from the lactating bats were unavailable for fat extraction.

[b]At least $p < 0.02$ vs all groups.

ing (not shown). There was no difference in cortisol between postprandial (107 ± 11 ng/mL) and fasted (122 ± 11 ng/mL) lactating bats (not shown).

Total body fat, expressed as a fat index (g fat/g lean dry mass), significantly increased after feeding in lactating bats, but not in second trimester pregnant animals (Table 2). The fat index was significantly greater in pregnant than lactating bats. Plasma leptin was significantly, but only weakly correlated ($r^2 = 0.26$) with fat index in lactating bats, and was not correlated with fat index when bats of all reproductive conditions were included in the analysis ($r^2 = 0.08$).

Discussion

Leptin restores gonadal function in experimentally starved mice *(9)*, restores fertility in obese female mice *(16)*, and accelerates the onset of puberty in mice *(17)*. Thus, we hypothesized that leptin would be high during periods of reproductive activity in bats. Highest irLeptin levels were observed in late pregnant bats, and the lowest levels were seen during lactation. The concentration of irleptin rose between the second and third trimesters of pregnancy. The apparent relationships between leptin and reproductive condition suggest a potential new role for this peptide, as a factor possibly contributing to the maintenance of pregnancy.

The basis for the higher leptin levels observed during pregnancy is unknown. Nightly food consumption increases during pregnancy in *M. lucifugus (18)*, and the present study

indicates that body fat also increases during this time. The weak, but significant correlation between fat index and leptin across all reproductive conditions in female *Myotis* is consistent with the well-known relationship between adiposity and circulating leptin in other species *(19,20)*. The weakness of the correlation, however, suggests that factors other than body adiposity are important in determining circulating leptin levels during pregnancy, at least in *Myotis* (*see* Note Added in Proof).

Small mammals require an enormous intake of energy to support the heat production needed to compensate for their high surface area/volume ratio, even during the relatively warm summer months in the northeastern United States. This is especially difficult during lactation, the most energetically demanding stage of a female mammal's life cycle. Thus, we predicted that leptin levels would decline during lactation, since leptin is associated with suppression of feeding, and lactation in *M. lucifugus* is associated with hyperphagia *(18,21)*. Our results support this hypothesis, since leptin decreased by roughly 60% between late pregnancy and lactation. In addition, lactation is well known to result in decreased fertility in mammals *(22)*. It is possible that the decline in circulating leptin during lactation may contribute to infertility during this time, just as a decrease in leptin may contribute to irregular estrous cyclicity in rodents *(9)*. *M. lucifugus*, however, breeds once per year in autumn and delays ovulation until the spring. Alternatively, therefore, high leptin levels may inhibit lactation, a hypothesis that could be tested by infusing leptin into lactating bats. Although the body fat index declined during lactation, the correlation between leptin and fat index during this period was not significant. Thus, factors other than changes in body fat likely account for part of the decrease in leptin seen during this time.

irLeptin was considerably higher in bats than reported for other species (e.g., *20,23*). In laboratory rodents subjected to a 3-d fast, adipose cell leptin mRNA increased soon after refeeding *(24)*. A 3-d fast in a small mammal, even at room temperature, constitutes a severe energetic stress *(22)*. Thus, we sought to determine if the normal daily fast encountered in the wild in *M. lucifugus* would also result in changes in leptin. We found that circulating irLeptin was higher in postprandial bats than in bats that had been fasting. However, a higher frequency of blood sampling (in the presence or absence of food) will be necessary to conclude that this relationship is the direct result of changes in energy status or postprandial metabolic cues (e.g., insulin). For example, evidence for diurnal changes in leptin exists in mice and humans *(9,23)*. Similar diurnal changes in leptin, if they occur in bats, could operate independently of energy status. Nonetheless, these results suggest that circulating leptin may be correlated with the fed/fasted state in free-ranging bats, and lends support to the hypothesis that a decrease in leptin is an adaptation to starvation *(9)*. These results extend this hypothesis to include the short-term, daily fasts that occur in nature. By contrast, however, it has been reported that leptin was similar in the blood of human volunteers 1–2 h after a midday meal and after an overnight fast *(20)*, and therefore, the role of leptin in acute fasting is still unsettled. Nonetheless, we believe that the paradigm used in the present study that relies on an animal's natural feeding cycle and diet can yield important clues to the role of circulating leptin in adaptation to energy scarcity.

The lack of significant changes in hypothalamic NPY in *Myotis* before and after feeding suggests that NPY may be less important in the control of energy balance in nonobese, free-ranging animals allowed to feed without constraints on food choice or availability than it appears to be in laboratory rodents. This is consistent with the recent finding that NPY-deficient mice do not show gross abnormalities in metabolism, body mass, feeding behavior, or sensitivity to leptin *(5)*. This conclusion should be tempered, however, by the caveat that intrahypothalamic distribution of NPY was not measured in the present study. As an example, normal (nonobese) laboratory rats trained to feed on a restricted schedule have a selective increase in paraventricular NPY just prior to feeding *(25)*. Changes in the intrahypothalamic distribution of NPY have been reported in *M. lucifugus* during different seasons of the year *(26)*. Thus, it is possible that redistribution of NPY within the hypothalamus may occur on a daily basis in *Myotis*, and such changes may not necessarily reflect an overall change in content.

The present results support an anorexogenic role for CRH, since acute fasting was associated with a significant drop in CRH content. Further analyses with increased sample size, as well as monitoring the immunocytochemical distribution of CRH in the hypothalamus of *Myotis*, may shed additional light on the strength of the relationship between CRH and fasting. Nevertheless, the decrease in CRH does not necessarily reflect increased hypothalamic/pituitary activity, since plasma cortisol levels (which peak around early evening in *Myotis*; *27*) did not change after feeding. It is important to note that it is premature at this stage to conclude that the changes in CRH, NPY, and leptin are functionally correlated. Future studies are needed to clarify the contributions of fasting and reproductive state on the expression of each of these peptides.

A disadvantage of the bat model is that homologous assays are not yet available for peptide hormones. Thus, each hormone assay was validated for *Myotis*. Serial dilutions of semipurified hypothalamic extracts or unextracted plasma competed for antibody binding in parallel with the authentic standards. irLeptin was measured with a human RIA kit, because in our experience, Chiropteran peptide hormones are most readily detectable with human-based RIAs and IRMAs *(27,28)*. To our knowledge, this is the first report of any species in which significant cross-

reactivity with antihuman leptin has been observed. Chiropteran plasma also cross-reacts with anti-(nonhuman) primate leptin, but not with antimouse leptin (unpublished observations). A second drawback is that during the course of each daily fast, a small degree of dehydration may be expected *(29)*, and this could conceivably influence neuroendocrine events related to neuropeptide synthesis and secretion.

The aforementioned caveats notwithstanding, we conclude that the little brown bat *(M. lucifugus)* is an important animal model useful for the study of central, endocrine, and metabolic control of energy balance. In addition, spontaneous fasting in the wild is associated with a decrease in circulating leptin and hypothalamic CRH, but not (total) NPY. We suggest that leptin may play a role in maintenance of pregnancy and lactation. It will be interesting to determine, for example, whether *ob/ob* mice, in whom fertility is restored by repeated injections of leptin *(16)*, can carry pregnancy to term if leptin injections are discontinued. It will also be interesting to determine if leptin administration inhibits lactation.

Materials and Methods

Study Species

M. lucifugus, the little brown bat, is an insectivorous species native to North America. The first of two nightly feeding bouts begins at ~2000 h and ends when the bats return to the roost at ~2330 h *(18)*. A second bout of feeding occurs between 0200 and 0500 h. Cessation of the second feeding is followed by a 15–16 h daily fast. Bats were collected at random immediately on returning from the first feeding and, in some cases, just prior to the first feeding. Female bats were captured from a maternity colony in New Hampshire using a harp trap suspended from the ridge pole *(30)*, and were pregnant or lactating at the time of capture (June 10–July 17, 1996). Occasional male bats and "nonreproductive" females were also collected.

Immediately after capture, bats were weighed and decapitated without anesthesia. Hypothalami were immediately dissected and frozen on dry ice. Plasma was collected into capillary tubes, centrifuged, and frozen for future RIA. Stomachs were dissected and the contents visually inspected to confirm the postprandial/fasted state. Pregnancy was assessed by abdominal palpation. Lactation was verified by manual palpation of the nipples and expression of milk. These procedures were approved by the Boston University Institute Animal Care and Use Committee.

Neuropeptide Analyses

Hypothalami were frozen on dry ice at the site of capture. On return to the laboratory, hypothalami were heated at 90°C for 5 min in 1 *N* acetic acid/0.1 *N* HCl with 5 μg/mL pepstatin, and then homogenized for 30 s. An aliquot of the homogenate was used for protein determination (mean protein content, 0.6 ± 0.1 mg). The soluble acid extract was chromatographed over C18 Sep Pak columns to purify further NPY and CRH. The elution buffer was acetonitrile (75%)/TEAF (triethylamine/formic acid; 25%). Recoveries of NPY and CRH were ~90%. Eluates were dried in a Speed Vac prior to radioimmunoassay. Porcine NPY standard (courtesy of J. Rivier, The Salk Institute) and rabbit antihuman NPY (courtesy of H. Spies, Oregon Regional Primate Center) at a final dilution of 1:20,000 were used for RIA of NPY. Rat/human CRH standard and rabbit anti-rat/human CRH (rC68; both courtesy of Rivier) were used to detect CRH as previously described *(31)*. Iodinated tracers were from New England Nuclear (Boston, MA).

Circulating Hormone Assays

Leptin was determined in 5 μL unextracted plasma using a leptin RIA kit (Linco, Inc., St. Louis, MO). Parallelism with the human leptin standard was determined using two separate pools of plasma from five bats each. Cortisol, the predominant glucocorticoid in *M. lucifugus (27)*, was determined in 5 μL unextracted plasma *(27)*.

Miscellaneous

Body fat was determined by extraction in a Soxhlet apparatus using a 3:1 mixture of ethanol/petroleum ether as the solvent *(32)*. Briefly, each carcass was dried to a constant mass, and then extracted and dried to a new constant mass. Fat index was defined as fat mass divided by lean dry mass. Data were analyzed by 1 factor analysis of variance and, where appropriate, by Mann-Whitney U-test. Data from male bats were not included in statistical analyses owing to low population size. Linear regression and statistical analyses were performed using Prism Software from GraphPad, Inc.

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Note Added in Proof

Since submission of this paper, leptin levels have been reported to rise during pregnancy in women (Butte et al., 1997, *J. Clin. Endocrinol. Metab.* **82,** 585–589; Masuzaki et al. 1997, *Nature Med.* **3,** 1029–1033), and leptin is secreted by the placenta (Masuzaki et al.).

References

1. Stanley, B. G., Lanthier, D., Chin, A. S., and Leibowitz, S. F. (1989). *Brain Res.* **501,** 32–36.
2. Zarjevski, N., Cusin, I., Vettor, R., Rohner-Jeanrenaud, F., and Jeanrenaud, B. (1993). *Endocrinology* **133,** 1753–1758.
3. Billington, C. J., Briggs, J. E., Harker, S., Grace, M., and Levine, A. S. (1994). *Am. J. Physiol.* **266,** R1765–R1770.
4. Sanacora, G., Kershaw, M., Finkelstein, J. A., and White, J. (1990). *Endocrinology* **127,** 730–737.
5. Erickson, J. C., Clegg, K. E., and Palmiter, R. D. (1996). *Nature* **381,** 415–417.
6. Erickson, J. C., Hollopeter, G., and Palmiter, R. D. (1996). *Science* **274,** 1704–1707.
7. Rothwell, N. J. (1990). *Neurosci. Biobehav. Rev.* **14,** 263–271.
8. Rohner-Jeanrenaud, F., Walker, C. D., Greco-Perotto, R., and Jeanrenaud, B. (1989). *Endocrinology* **124,** 733–739.
9. Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., and Flier, J. S. (1996). *Nature* **382,** 250–252.
10. Schwartz, M. W., Baskin, D. G., Bukowski, T. R., Kuijper, J. L., Foster, D., Lasser, G., Prunkard, D. E., Porte, D., Jr, Woods, S. C., Seeley, R. J., and Weigle, D. S. (1996). *Diabetes* **45,** 531–535.
11. Mercer, J. G., Hoggard, N., Williams, L. M., Lawrence, C. B., Hannah, L. T., Morgan, P. J., and Trayhurn, P. (1996). *J. Neuroendocrinology* **8,** 733–735.
12. Smith, F. J., Campfield, L. A., Moschera, J. A., Bailon, P. S., and Burn, P. (1996). *Nature* **382,** 307.
13. Stephens, T. W., Basinski, M., Bristow, P. K., Bue-Valleskey, J. M., Burgett, S. G., Craft, L., Hale, J., Hoffmann, J., Hsuing, H. M., Kriauciunas, A., MacKellar, W., Rosteck, P. R., Jr, Schoner, B., Smith, D., Tinsley, F. C., Zhang, X.-Y., and Heiman, M. (1995). *Nature* **377,** 530–532.
14. Campfield, L. A., Smith, F. J., Guisez, Y., Devos, R., and Burn, P. (1995). *Science* **269,** 546–549.
15. Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Lallone, R. L., Burley, S. K., and Friedman, J. M. (1995). *Science* **269,** 543–546.
16. Chehab, F. F., Lim, M. E., and Lu, R. (1996). *Nat. Genet.* **12,** 318–320.
17. Chehab, F. F., Mounzih, K., Lu, R., and Lim, M. E. (1997). *Science* **275,** 88–90.
18. Anthony, E. and Kunz, T. H. (1977). *Ecology* **58,** 775–786.
19. Schwartz, M. W., Peskind, E., Raskind, M., Boyko, E. J., and Porte, D., Jr (1996). *Nat. Med.* **2,** 589–593.
20. Ma, Z., Gingerich, R. L., Santiago, J. V., Klein, S., Smith, C. H., and Landt, M. (1996). *Clin. Chem.* **42,** 942–946.
21. Burnett, C. D. and Kunz, T. H. (1982). *J. Mammology* **63,** 33–41.
22. Bronson, F. H. (1989). *Mammalian Reproductive Biology.* University of Chicago Press, Chicago.
23. Sinha, M. K., Ohannesian, J. P., Heiman, J. L., Kriauciunas, A., Stephens, T. W., Magosin, S., Marco, C., and Caro, J. F. (1996). *J. Clin. Invest.* **97,** 1344–1347.
24. Freiderich, R. C., Lollman, B., Hamann, A., Napolitano-Rosen, A., Kahn, B. B., Lowell, B. B., and Flier, J. S. (1995). *J. Clin. Invest.* **96,** 1658–1663.
25. Kalra, S. P., Dube, M. G., Sahu, A., Phelps, C. P., and Kalra, P. S. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 10,931–10,935.
26. Laemle, L. K and Cotter, J. R. (1992). *J. Comp. Neurol.* **316,** 447–458.
27. Widmaier, E. P., Harmer, T. L., Sulak, A. M., and Kunz, T. H. (1994). *J. Exp. Zool.* **269,** 442–449.
28. Widmaier, E. P., Gornstein, E. R., Hennessey, J. L., Bloss, J. M., Greenberg, J. A., and Kunz, T. H. (1996). *Am. J. Physiol.* **271,** R1101–R1106.
29. Kurta, A., Bell, G. P., and Nagy, K. A. (1989). *Can. J. Zool.* **67,** 2468–2472.
30. Kunz, T. H. and Kurta, A. (1988). In: *Ecological and Behavioral Methods for the Study of Bats.* Kunz, T. H. (ed.), Smithsonian Institution Press: Washington, DC, pp. 1–30.
31. Widmaier, E. P., Lim, A. L., and Vale, W. (1989). *Endocrinology* **124,** 583–590.
32. Kunz, T. H., Burnett, C. D., and Wrazen, J. R. (1997). *Ecoscience*, in press.