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PHENOTYPIC EFFECTS OF LEPTIN IN AN ECTOTHERM: A NEW TOOL TO STUDY THE EVOLUTION OF LIFE HISTORIES AND ENDOTHERMY?

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Summary

Leptin is a hormone that regulates energy expenditure and body mass in mammals, and it has attracted considerable attention because of its potential in treating human obesity. Comprehensive data from both pathological and non-pathological systems strongly support a role for leptin in regulating energy metabolism, in thermoregulation and in regulating the onset of puberty. We report here that daily injections of recombinant murine leptin in fence lizards (*Sceloporus undulatus*) produce phenotypic effects similar to those observed when leptin injections are given to mice. Lizards injected with leptin had body temperatures 0.6 °C higher, ate 30 % less food and showed a 14 % reduction in activity rates, and females showed a 2.5-fold increase in resting metabolic rates, compared with lizards injected with vehicle only (phosphate-buffered saline). We also detected native lizard leptin using an immunoassay. Our results indicate that leptin is expressed in ectotherms and may be conserved both functionally and structurally. In the wake of unprecedented research activity on the role of leptin as a cause of, and potential treatment for, human obesity, we believe that other applications of leptin research have been ignored. For example, the response of lizards to leptin injection in our study has important implications for two broad areas of research in evolutionary biology: the evolution of age at first reproduction and of endothermy. We argue that research in these areas, previously limited to comparative approaches, may now benefit from experimental manipulations using leptin.

Key words: leptin, energy expenditure, obesity, thermoregulation, fence lizard, *Sceloporus undulatus*, phenotypic engineering.

Introduction

Leptin is the 16 kDa hormone product of the mouse obesity (*ob*) gene (Halaas et al., 1997). Mice that are homozygous for a mutation of the *ob* gene (*ob*/*ob*) do not produce leptin and, consequently, suffer from obesity and diabetes, with similar effects to those observed in victims of morbid human obesity syndrome (Boston et al., 1997). One of the primary functions of leptin appears to be as a lipostat in the homeostasis of fat stores (adiposity); however, extensive research into obesity has revealed that leptin has many other phenotypic effects. Most are correlated with adiposity, including the regulation of metabolic rate, feeding and activity rates, body temperature and the onset of puberty (Flier, 1997). Interestingly, a correlation between adiposity and the onset of reproduction (puberty) in mammals has long been recognized and a hormonal signal sought (Kennedy and Mitra, 1963; Frisch, 1972; Frisch and McArthur, 1974; Frisch et al., 1975; Merry and Holehan, 1979; Van der Spuy, 1985; Bronson, 1988; Beunen et al., 1994). However, the hormonal signal(s) underlying the correlation has never been identified.

Recent experiments suggest that leptin may be important in regulating the onset of maturation by mediating hormonal signals of adiposity (Ahima et al., 1997; Strobel et al., 1998). For example, homozygous obese female mice (*ob*/*ob*) are sterile, but their sterility can be reversed by the administration of human recombinant leptin (Barash et al., 1996). Similarly, starvation-induced reductions in circulating levels of native leptin and the consequent delay of ovulation in female mice can be treated by the administration of leptin (Ahima et al., 1996). More significantly, in normal mice, the administration of leptin decreases age at maturity with no apparent detrimental effects (Chehab et al., 1996). Overall, data across a wide range of taxa suggest that adiposity is causally related to the onset of maturity and that, in mammals, leptin is an important metabolic signal in this link.

Until recently, research on leptin has been restricted to mammalian systems. Consequently, we do not yet know whether leptin is present in other vertebrates. Other metabolic regulators that interact with leptin in modulating food intake in mammalian systems are very broadly distributed phylogenetically (e.g. neuropeptide Y, NPY; corticotropin-releasing hormone, CRH; Peptide YY). Therefore, it seems likely that leptin is also broadly distributed phylogenetically, and evidence to that effect is accumulating given the discovery of leptin in chickens (Taouis et al., 1998; Ashwell et al., 1999). While evidence for a broad distribution in endotherms is expanding, there is still no evidence that ecotherms also have leptin. Many ectotherms undergo a tightly regulated seasonal cycling of fat reserves associated with reproduction and hibernation, presumably requiring a sophisticated lipostatic system. However, it is not known whether such regulation is provided by leptin. Furthermore, even if ectotherms do have leptin and it functions as a lipostat, it is unclear whether the correlated phenotypic effects (especially metabolic, thermoregulatory and reproductive) would be similar.

Using lizards as a representative ectotherm, we tested the hypothesis that lizards have leptin. The high sequence similarity of leptins among different taxa from which it has already been isolated suggests strong conservation of structure and function; we therefore tested for the presence of leptin in lizards in two ways. First, we measured the phenotypic effects on lizards of injecting recombinant murine leptin. Second, we detected native lizard leptin *via* immunoblot, using polyclonal antibodies against mouse leptin.

Materials and methods

Murine leptin injection

Twenty fence lizards (*Sceloporus undulatus* Boulenger), collected from Arthur Co., Nebraska, USA, were randomly assigned to either a control (PBS; body mass 3.5±0.18 g) or treatment (LEP; body mass 3.45±0.14 g; means ±2 S.E.M.) group. Each lizard was housed individually in a 40 l aquarium equipped with a non-light-emitting heating element at one end providing a thermal gradient between 28.0 and 36.0 °C during a 10 h:14 h photoperiod (measured using copper models; Niewiarowski and Roosenburg, 1993). Night-time temperatures were approximately 20 °C. Aquaria were arranged five to a shelf on two racks of shelves inside a temperature-controlled room. Treatments were dispersed across shelves, with shelves treated as blocks and accounted for as a random effect in all statistical analyses. Each aquarium was stocked daily with five crickets to serve as an *ad libitum* food source (Niewiarowski, 1995). At 08:00 h each morning during a 14 day experimental period, PBS lizards were given a 150 µl intraperitoneal injection of phosphate-buffered saline, while LEP lizards were given a 150 µl intraperitoneal injection of 10 µg g^{-1} body mass of recombinant murine leptin (Peprotech, Rocky Hill, NJ, USA) dissolved in phosphate-buffered saline. We used 10 µg g^{-1} leptin because experiments with mice had shown maximal effects at this dose (Pelleymounter et al., 1995). Following injection, lizards were returned to their aquaria.

On each day, prior to injection, each lizard was scored for activity (buried beneath sand/debris or active on the surface), then captured and cloacal temperature (T_b) measured and mass recorded. We counted and weighed the crickets remaining uneaten in each aquarium from the previous day's introduction of five crickets. Finally, at 12:00 h and 17:00 h, lizards were again scored for activity and their cloacal temperatures recorded. On days 13 and 14, we measured oxygen consumption and carbon dioxide production of each lizard during a 2 h period using a Sable System TR-3 flow-through respirometry system (1 p.p.m. CO_2 and 0.001 % O_2 detection limits). At the end of day 14, we gave lizards an overdose of MS222 and collected a blood sample by heart puncture. Blood smears were stained (Wright's solution), and the number of white blood cells was compared between PBS and LEP lizard smears. We removed and weighed abdominal fat bodies, dried the carcasses and extracted whole-body lipids with a Soxhtet extraction unit. Dependent variables were ln-transformed when a Shapiro–Wilk test rejected a null hypothesis of normality. We analyzed body temperature variation using a double repeated-measures multivariate analysis of variance (MANOVA) because T_b observations across time of day and experimental day for each lizard were not independent (Winer, 1971). We analyzed joint variation in mass change (final mass minus initial mass), total mass, the number of crickets eaten and percentage activity (the number of periods scored active divided by the total number of periods scored) using MANOVA. Variation in metabolic rate, respiratory quotient (RQ) and fat body mass was analyzed by ANOVA.

Native lizard leptin immunoblot

Tissues were excised and homogenized in 50 mmol l^{-1} Hepes, pH 7.4, at 10 % (w/v) ratios. Homogenates were centrifuged at 10 000 ***g*** for 5 min and the supernatant removed. Supernatants were assayed for protein concentration (Smith et al., 1985), solubilized in Laemmli sample buffer (Laemmli, 1970) and electrophoresed through duplicate 15 % Tricine gels (Schagger and von Jagow, 1987). One duplicate gel was stained in Coomassie Brilliant Blue (0.05 % Coomassie Brilliant Blue R250, 10 % methanol, 10 % acetic acid) and the other was electroblotted to polyvinylidene difluoridine (PVDF) membrane. Blots were blocked in 5 % non-fat dry milk in phosphate-buffered saline (PBS) overnight, washed (three times for 10 min) in PBS, then incubated in 1:1000 polyclonal rabbit anti-mouse leptin antibody (Chemicon, Temecula, CA, USA) in a carrying solution of 0.01 % bovine serum albumin in PBS at 4 °C overnight with agitation. Blots were again washed with three changes of PBS (10 min each) and incubated with 1:5000 dilution of horseradish-peroxidase-labeled goat anti-rabbit antibody (Pierce, Rockford, IL, USA) for 5 h. Blots were washed with PBS a final time (three times for 10 min), developed with a chemiluminescent substrate (Supersignal, Pierce) and exposed to film.

Results

Murine leptin injection

LEP and PBS lizards did not differ in mean T_b values across days of the experiment (Fig. 1; repeated measure='day', Wilk's $\lambda_{10,26}$=0.38, P=0.16), but LEP lizards had higher average T_b values than PBS lizards as a function of time of day (repeated measure='time of day', Wilk's $\lambda_{55,22}$=0.0003, P=0.05; main effect='treatment', Wilk's $\lambda_{11,4}$=0.0473, P=0.03). Block was also significant (Wilk's $\lambda_{33,12}$=0.0029, P=0.05), but sex was not, and all other interactions were not significant. Because 'time of day' was significant, we analyzed each time separately (Winer, 1971). Separate analyses revealed

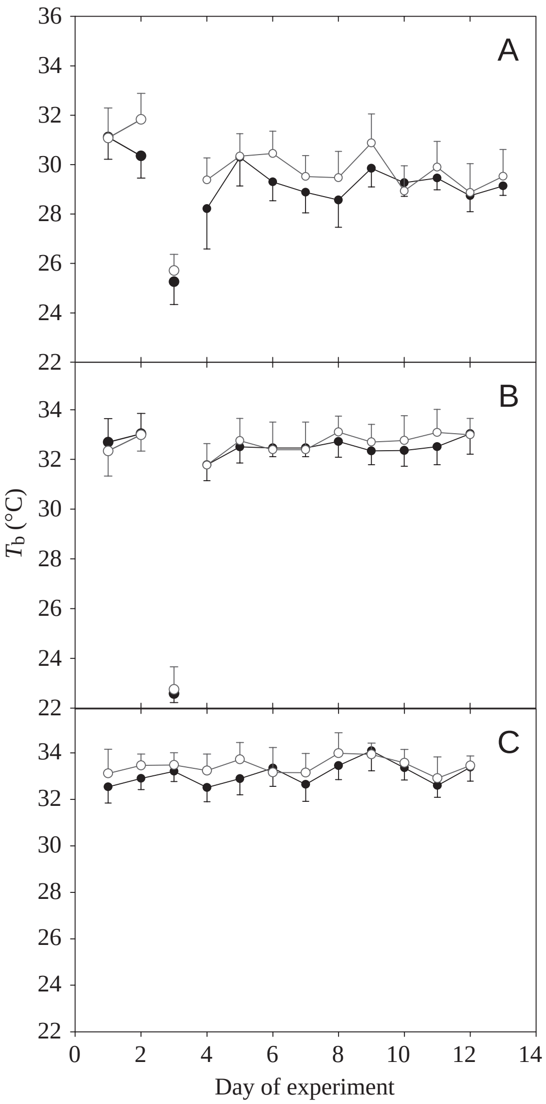

Fig. 1. Mean daily body temperatures (T_b) of saline-injected (PBS) and leptin-injected (LEP) lizards over the course of the experiment. Values displayed are means ±2 S.E.M., N=10 for each group. (A) T_b at 08:00 h; (B) T_b at 12:00 h; (C) T_b at 17:00 h. Filled and open symbols are for PBS and LEP lizards, respectively. The break in the T_b trace was caused by a power failure to the heat lamps on day 3.

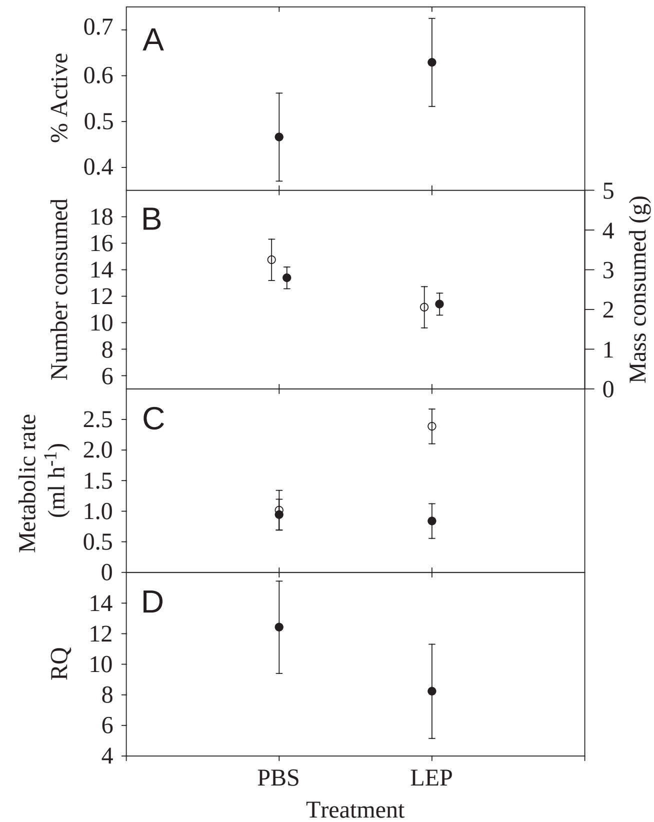

Fig. 2. (A) Percentage activity (the ratio of the number of times a lizard was observed above ground to the total number of times it could have been above ground) ($F_{1,14}$=5.84, P=0.0298). (B) Total number (open symbols; $F_{1,14}$=10.49, P=0.006) and mass (filled symbols; $F_{1,14}$=11.65, P=0.004) of crickets consumed by each lizard over the entire experimental period; symbols are offset for clarity. (C) Metabolic rate (rate of oxygen consumption) of females (open symbols) and males (filled symbols) estimated as total oxygen consumption. (D) Ranked respiratory quotient (rate of O_2 consumption/rate of CO_2 production). Values displayed in all panels are least-square means ±2 S.E.M., N=5; i.e. responses have been adjusted for all sources of variance in the model except for the treatment effect (LEP or PBS). LEP, leptin-injected lizards; PBS, saline-injected lizards.

no significant differences between LEP and PBS T_b at any single time of day, indicating that the correlations between effects at different times are driving the significance of the overall model (Keppel, 1982). T_b values of LEP lizards seemed to be higher than those of PBS lizards when the two differed (Fig. 1), so we used a runs test (Sokal and Rohlf, 1981) to test the hypothesis that mean T_b values of LEP lizards were equally likely to be higher than as lower than T_b values of PBS lizards. LEP lizards had higher T_b values than PBS lizards more often than expected by chance at 08:00 h (Fig. 1A) (runs test, N=12, r=3, P<0.05), but not at 12:00 h (Fig. 1B) and 17:00 h (Fig. 1C). The dip in temperatures for 08:00 h and 12:00 h on day 3 was due to a power failure affecting the heating elements.

MANOVA on activity rate, number and mass of crickets consumed and mass change of lizards comparing LEP with PBS lizards showed a significant difference (Wilk's $\lambda_{20,37}$=0.043, P=0.0021). Subsequent ANOVAs showed that PBS and LEP lizards did not differ in mass change (+0.62±0.15 g for PBS; +0.61±0.22 g for LEP), but did differ significantly with respect to the other dependent variables. Female LEP lizards had an approximately twofold higher rate of oxygen consumption than female PBS lizards (ANOVA; $F_{1,12}$=10.2, P=0.008) (Fig. 2C). Ranking of respiratory quotient (raw RQs were not normally distributed) showed a tendency (not significant at P<0.05) to be lower for LEP than for PBS lizards (ANOVA; $F_{1,16}$=3.68, P=0.073) (Fig. 2D), suggesting preferential fat metabolism. Fat body mass and white blood cell counts did not differ between PBS and LEP

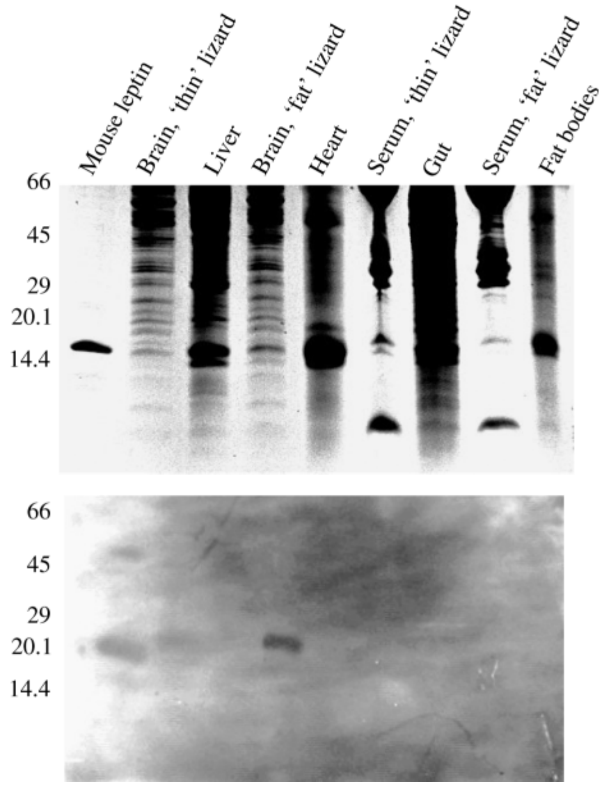

Fig. 3. Coomassie-stained SDS–PAGE gel (upper panel) and immunoblot (lower panel) of *Sceloporus undulatus* tissues. Tissues are labeled above each lane, and lane assignments are identical for the gel and blot. Molecular mass (kDa) is indicated on the vertical axis. Protein loaded: 0.5 μg for mouse leptin, 40 μg for brain, 50 μg for heart, liver and gut, and 20 μg for fat bodies. The mass:length ratio of the 'fat' lizard was 15 % higher than that for the 'thin' lizard.

lizards (data not shown). LEP lizards not only had higher T_b values on average but also had significantly higher activity rates (Fig. 2A) and significantly lower feeding rates than PBS lizards (Fig. 2B). Unexpectedly, leptin injection also affected the metabolic variables of lizards (Fig. 2C,D). In spite of the higher metabolic rates and lower feeding rates of LEP compared with PBS lizards, there was no significant difference in weight gain.

Native lizard leptin

Polyclonal antibodies against mouse leptin recognize a protein of similar molecular mass in the brain of fence lizard (Fig. 3). An immunoreactive band that co-migrates with recombinant mouse leptin is evident in brain homogenates, but not in other tissues, including serum and the sites of leptin production in mammals (adipose tissue; Halaas et al., 1997) and in chicken (liver; Taouis et al., 1998). We do not interpret this result as brain being the source of leptin in lizards, however. Rather, we suggest it is more likely that the mouse antibody cross-reacts relatively weakly with lizard leptin, and only in the brain, where leptin bound to the leptin receptor is relatively concentrated, is the band visible. Regardless, these results indicate that *S. undulatus* expresses a leptin-like protein.

Discussion

Models of leptin function are increasing in complexity with each new study (Gillis, 1997). Although the full details of its mechanism and action remain to be worked out, it is clear that leptin is a central lipostatic hormone in mammals. Furthermore, in mammals, the data now also implicate leptin as an important signal indicating that fat stores are sufficient to commence reproduction (Boston et al., 1997; Flier, 1997). Although the functions of leptin have been elucidated exclusively from mammalian systems, the relationship between fat stores and reproductive maturity has been observed in many vertebrates (Silverstein et al., 1997; Yannakopoulos et al., 1995; Derickson, 1976; Benabib, 1994). Furthermore, the discovery of leptin in chickens (Taouis et al., 1998) is probably indicative of a very broad phylogenetic distribution. Our data from leptin injections in lizards are surprisingly consistent with experiments on mice (Pelleymounter et al., 1995); we have demonstrated that intraperitoneal injection of murine leptin in lizards produces most of the short-term physiological effects observed in mice.

In view of the significant increase in activity and decrease in feeding rate but lack of significant decrease in body mass of LEP compared with PBS lizards, our results may be most appropriately compared with experimental results from lean mice. Pelleymounter et al. (1995) and Halaas et al. (1995) found that body mass was not responsive or only weakly responsive to leptin injection in lean (wild-type) mice, even though most of the other variables measured (i.e. body temperature, activity and metabolic rate) did respond. Furthermore, the substantially lower metabolic (approximately 10-fold) rates of ectotherms compared with endotherms impacts upon our results. The likelihood of detecting mass changes on the basis of energy differences accrued from metabolic and feeding rate differences is low considering the short experimental period (13 days).

The role of leptin in regulating reproduction was first observed in the *ob*/*ob* mouse mutation system used to study obesity. Sterility is one phenotypic consequence of the *ob*/*ob* mutation; gonadal development is normal, but ovulation fails to occur and mice never enter into oestrus. Chehab et al. (1996) showed that, in addition to reversing obesity, injecting leptin into female *ob*/*ob* mice induces ovulation and reproductive maturation. Barash et al. (1996) demonstrated a regulatory role for leptin in *ob*/*ob* male reproduction as well; leptin injection increases testis mass, seminal vesicle mass and sperm count. The regulatory role of leptin in reproduction is not limited to the pathological mutation systems used to study obesity in mice. For example, serum leptin levels increase during puberty in human males (Mantazoros et al., 1997) and in pigs (Qian et al., 1999), and the onset of menarch in human females is inversely related to serum leptin concentrations (Matkovic et al., 1997). Ahima et al. (1997) have also shown experimentally that leptin injection accelerates the onset of puberty in normal female mice. A wide range of comparative and experimental data from a variety of taxa confirm the role of leptin in the regulation of reproductive maturity in mammals. We have demonstrated that virtually all the short-term effects of leptin injection observed in mice are also observed in lizards. By extension, we expect similar long-term effects (earlier age at maturity) observed in mice and other mammals to be manifested in lizards. Such a result would make it possible to use leptin to manipulate age at maturity in lizards and, presumably, in a wide range of taxa and experimental contexts.

The results from our experiments are similarly provocative in an entirely different research context, the evolution of endothermy. The approximately 2.5-fold increase in oxygen consumption of female LEP lizards compared with PBS lizards (Fig. 2C) was unexpected and, we believe, unprecedented. In contrast to mammals, lizards and other ectotherms do not regulate their metabolic rate to maintain a constant T_b. The cellular basis of the metabolic response in our experiments is unclear; however, in both ectothermic and endothermic vertebrates, a large percentage of standard metabolic rate is generated by proton leak across the mitochondrial inner membrane. It has been suggested that uncoupling proteins (UCPs) may be one source of this mitochondrial proton leak (Flier and Lowell, 1997; Gura, 1997). Recently, it has been shown that the expression of uncoupling protein-1 (UCP-1) is stimulated by leptin injection (Scarpace et al., 1997). Another UCP (UCP-2), with 56 % amino acid sequence homology to UCP-1, was recently shown to be ubiquitously expressed in mammalian tissues and to be more strongly related to mitochondrial membrane potential than to UCP-1 (Fleury et al., 1997).

It is possible that the thermogenic and metabolic effects associated with exogenous leptin administration (e.g. Pelleymounter et al., 1995) are related to proton leakage rates modulated in some way by UCPs. The 2.5-fold increase in standard metabolic rate of female LEP compared with PBS lizards is intriguing because it represents a significant fraction of the typically five- to tenfold lower standard metabolic rates of reptiles relative to mammals (Else and Hulbert, 1987). In other words, by manipulating levels of leptin, which has been linked to the regulation of the proton leak (a correlate of standard metabolic rate differences between endotherms and ectotherms), we could account for 25–50 % of the difference in standard metabolic rates between ectotherms and endotherms of a given body size. Our study suggests that manipulation of ectotherm metabolic rates using exogenous leptin could help to determine whether the differences in metabolic rates between ectotherms and endotherms are a result of differences in membrane surface area and composition (Rolfe and Brand, 1997; Brand, 1990; Hulbert and Else, 1989; Else and Hulbert, 1987). Irrespective of the mechanism of proton leak, elucidating the difference in the characteristics of leptin between ectotherms and endotherms is likely to provide important insights into the cellular and molecular differences between ectothermy and endothermy.

Studying leptin in a phylogenetic context is a powerful approach not only for elucidating its phenotypic effects but also for understanding the structure and function of the hormone itself. Currently, the only non-mammalian sequence published is that of chicken leptin (Taouis et al., 1998). Identifying, cloning and sequencing leptin in other non-mammalian vertebrate taxa would allow analysis of the structural limitations of the hormone. Blocks of primary sequence that are conserved across evolutionarily distant taxa are likely candidates for the domains of the protein that are most important for its function. Without the alignments that can be generated with sequences from divergent taxa, and in the absence of a crystal structure, identifying candidate residues for site-directed mutagenesis is difficult. This approach (i.e. sequence alignment from divergent taxa) has yielded significant progress in functional studies of other proteins such as large Ca^{2+} channels (Takeshima et al., 1994).

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