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24-HOUR CHANGES IN ACTH, CORTICOSTERONE, GROWTH HORMONE, AND LEPTIN LEVELS IN YOUNG MALE RATS SUBJECTED TO CALORIE RESTRICTION

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Calorie restriction of young male rats increases plasma prolactin, decreases luteinizing hormone (LH) and testosterone, and disrupts their 24 h secretory pattern. To study whether this could be the consequence of stress, we examined the 24 h variations of plasma adrenocorticotropic hormone (ACTH) corticosterone, growth hormone (GH), leptin, and adrenal corticosterone. Rats were submitted to a calorie restriction equivalent to a 66% of usual intake for 4 weeks, starting on day 35 of life. Controls were kept in individual cages and allowed to eat a normal calorie regimen. Significantly lower ACTH levels were detected in calorie-restricted rats. Plasma corticosterone levels during the light phase of the daily cycle were significantly higher in calorie-restricted rats. Time-of-day variation in plasma ACTH and corticosterone levels attained significance in calorie-restricted rats only, with a maximum toward the end of the resting phase. The daily pattern of adrenal gland corticosterone mirrored that of circulating corticosterone; however, calorie restriction reduced its levels. Plasma ACTH and corticosterone correlated significantly in controls only. Calorie restriction decreased plasma GH and leptin, and it distorted 24 h rhythmicity. In a second study, plasma ACTH and corticosterone levels were measured in group-caged rats, isolated control rats, and calorie-restricted rats during the light phase of the daily cycle. Plasma ACTH of calorie-restricted rats was lower, and plasma corticosterone was higher, compared with isolated or group-caged controls. The changes in the secretory pattern of

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hormones hereby reported may be part of the neuroendocrine and metabolic mechanisms evolved to maximize survival during periods of food shortage.

Keywords Calorie restriction, ACTH, Corticosterone, Growth hormone, Leptin, Food anticipatory activity, Circadian rhythm

INTRODUCTION

Scarcity of food occurs recurrently in nature every year and can be experimentally reproduced by submitting animals to a calorie restriction. A calorie restriction ranging from a 25% to 50% of normal caloric intake (without deficiency in essential nutrients) has been often employed in aging studies, and this manipulation results in a slowing of the aging process in rodents (Masoro et al., 1992; Masoro, 2000; Masoro and Austad, 1996; Wanagat et al., 1999).

Under calorie restriction, plasma levels of several hormones are modified (Brogan et al., 1997; Chacon et al., 2002; Wronska et al., 1990). Among them, the hormones of the pituitary-gonadal axis have been extensively studied, mostly using single time-of-day sampling (Caprio et al., 2001; Clark et al., 1998; Sprangers and Piacsek, 1997). We recently reported that calorie restriction of young male rats brought about significant increase of prolactin and decrease of luteinizing hormone (LH) and testosterone, as well as disruption of their 24 h secretory patterning (Chacon et al., 2004). Therefore, some consider that food presentation and availability can serve as a synchronizer of the circadian system (Mistlberger, 1994).

In our earlier study, calorie restriction brought about a significant reduction in body weight, which was significant after the first week of the diet; the body weight of calorie-restricted rats was reduced to 62% of that of controls after 4 weeks of treatment (Chacon et al., 2004). Moreover, in calorie-restricted rats available food was consumed over a short span of time, followed by a span of starvation. Therefore, it is possible that restricted food availability is a stressor, as suggested by the finding of increased prolactin levels in our earlier study (Chacon et al., 2004). Indeed, the question remains as to whether this experimental paradigm of caloric restriction is accompanied by a hormone profile indicative of stress. Most data support such a role of reduced food availability (Armario et al., 1987). However, paradoxical reduction of basal plasma adrenocorticotropic hormone (ACTH) and enhancement of circulating corticosterone levels associated with hypoleptinemia have been observed after a 50% to 60% caloric restriction in rodents (Avraham et al., 2002). This finding prompted us to undertake the present study to assess the 24 h variations of plasma levels of ACTH, growth hormone (GH) leptin,

and corticosterone and of adrenal gland corticosterone in growing male rats.

MATERIALS AND METHODS

Animals and Experimental Design

Five week-old male Wistar rats were kept under standard conditions of controlled light (fluorescent cool white bulbs providing 100 lux intensity at the level of cages) of a 12:12 h light/dark schedule (light on at 08:00 h) and temperature (22°C ± 2°C). All experiments were conducted in mid-spring (April to May). Two studies were performed. In study 1, both calorie-restricted rats and normally fed controls were assessed. All animals were caged individually to avoid cannibalism among the calorie-restricted rats (Goonewardene and Murasko, 1995; Pugh et al., 1999). Control rats had free access to an equilibrated diet (AIN-93G, Dyets Inc., Bethlehem, Pennsylvania, USA) and water for 4 weeks. Calorie-restricted rats had daily access to 7 g of an unbalanced AIN-93G diet enriched in protein and low in fat and carbohydrate (Reeves et al., 1993) and water *ad libitum* for 4 weeks. This calorie restriction was equivalent to a 66% reduction of normal caloric intake, calculated by considering the average of food consumption of the control group. Presentation of the restricted calorie diet took place between 09:00 to 10:00 h daily, with the food being consumed over a short time of about 2 to 3 h. Groups of rats were killed by decapitation at 6 different time intervals, every 4 h, throughout a single 24 h span starting 1 h after light on (HALO), at 09:00 h. During the dark span, animals were killed under red dim light. Seven to 8 control as well as calorie-restricted animals were killed by decapitation at each one of the 6 time points. Blood was collected from the trunk wound in heparinized tubes and thereafter centrifuged at 1500 × g for 15 min. The plasma was collected and stored at −20°C until further analysis.

In a second study, three groups of rats were compared: calorie-restricted rats, normally fed controls individually caged, and normally fed controls caged in groups (4 to 5 animals per cage). The diet was as in study 1. After 4 weeks, groups of rats (7 per group) were killed by decapitation at three specific time points during the rest span (*i.e.*, at 1, 5, and 9 HALO). Blood was collected and processed as in study 1.

The care and use as well as all procedures involving animals were approved by the Institutional Animal Care Committee, Faculty of Medicine, Complutense University, Madrid. The experiments were conducted in accordance with the guidelines of the International Council for Laboratory Animal Science (ICLAS) and with the good practice

standards for biological rhythm research on laboratory animals as establish by the journal (Touitou et al., 2004).

Hormone Assays

Plasma prolactin, ACTH, and GH levels were determined by a homologous specific double antibody RIA, using materials kindly supplied by the NIDDK's National Hormone and Pituitary Program. Sensitivities of the RIAs were 45, 195, and 40 pg/mL for prolactin, ACTH, and GH using the NIDDK rat prolactin RP-3, rat ACTH-RP-1, and rat GH-RP-2, respectively. Intraassay coefficients of variation were 7%, 8.5%, and 7.4% for prolactin, ACTH, and GH, respectively. The interassay coefficients of variation were 9, 10, and 10%, respectively (Castrillón et al., 2001; Esquifino et al., 1999; Garcia Bonacho et al., 2000). Plasma levels of leptin (validated for rat leptin) and corticosterone as well as adrenal content of corticosterone were also determined by specific RIA methods as in previous studies (Giovambattista et al., 2000; Spinedi et al., 1991), their intraassay coefficients of variation being 6.5% and 5%, respectively; whereas, the interassay coefficient of variation was 11% and 9%, respectively. Results are expressed as ng/mL for prolactin, GH, and leptin, as pg/mL for ACTH, as μg/dL for plasma corticosterone, and as μg/adrenal for adrenal corticosterone.

Statistical Analysis

Statistical analysis of the data was carried out by a two-way factorial analysis of variance (ANOVA) or a one-way ANOVA, as stated. Generally, the factorial ANOVA included an assessment of the diet effect (*i.e.*, occurrence of differences in mean values between calorie-restricted and control groups), of time-of-day effects (the occurrence of daily changes), and of interaction between the two factors (diet and time, from which differences in timing and amplitude could be inferred). Post-hoc Tukey-Kramer's multiple comparisons tests from the a one-way ANOVA were then employed to determine which time point means were significantly different within each experimental group and to define the time of peaks. Curve estimation in regression analysis was made by using SPSS software, version 10.1 (SPSS Inc., Chicago, IL, USA). p values <0.05 were considered evidence for statistical significance.

RESULTS

Figure 1 (top) shows the 24 h changes in the plasma ACTH concentration of calorie-restricted and normally fed rats. Factorial ANOVA revealed both treatment and time were significant main effect factors

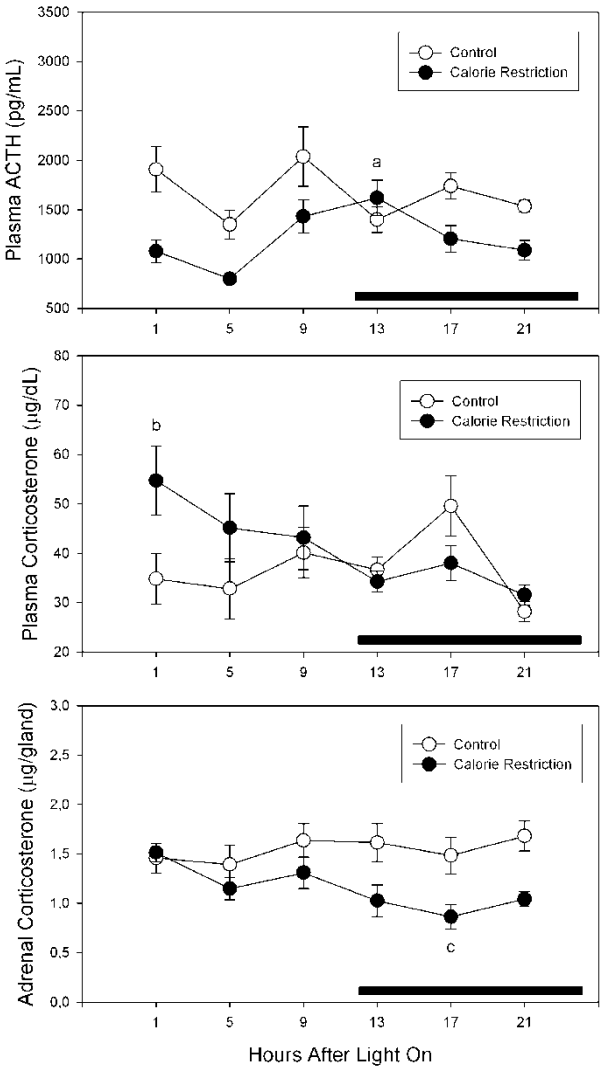

FIGURE 1 Effect of calorie restriction on 24 h changes in plasma ACTH (top), plasma corticosterone (middle), and adrenal gland corticosterone concentration (bottom) in male rats. Groups of 7 to 8 rats were killed by decapitation at 6 different times throughout a single 24 h cycle. Bar indicates scotophase duration. Results are the means ± SEM. Letters indicate the existence of significant differences between the time points means within each group by one-way ANOVA followed by a Tukey-Kramer's multiple comparisons test, as follows: $^{a}p < 0.05$ versus 1, 5, 17, and 21 HALO; $^{b}p < 0.05$ versus 21 HALO; $^{c}p < 0.01$ versus 1 HALO. For further statistical analysis, see text.

($F_{1,78} = 23.1$, $p < 0.00001$ and $F_{5,78} = 3.87$, $p < 0.004$); calorie restriction was associated with significantly decreased ACTH levels. A significant interaction "time × diet" ($F_{5,78} = 3{,}14$, $p < 0.05$) was also found, *i.e.*, time of day variations of ACTH concentration in control rats did not attain significance, whereas in calorie-restricted rats, a maximum toward

the end of the resting phase was apparent as shown by individual one-way ANOVA's (Figure 1, upper panel).

Figure 1 (middle) depicts the daily pattern of plasma corticosterone in calorie-restricted and normally fed rats. In the factorial ANOVA, only time of day was identified as significant main factor ($F_{1,78} = 2.45$, $p < 0.05$), and a significant interaction "time × diet" was found ($F_{5,78} = 2.39$, $p < 0.05$). In calorie-restricted rats a maximum of plasma corticosterone concentration occurred at the beginning of the resting phase; whereas, in control rats, time of day variations were not statistically significant (Figure 1, middle panel, one-way ANOVA). A closer inspection of the plasma corticosterone data indicates that values during the rest span of the daily cycle did differ between groups. A factorial ANOVA applied to the data of the three time intervals of the rat photophase (*i.e.*, 1, 5 and 9 HALO) indicated the calorie-restricted rats had higher plasma corticosterone levels than did the controls ($F_{1,38} = 5.25$, $p < 0.03$). Circulating corticosterone correlated with ACTH levels in control rats only. This correlation was best described by a log model with $r^2 = 0.262$, $b_0 = -41.4$, and $b_1 = 10.6$ ($F = 3.75$, $p = 0.02$). Such a correlation was not observed in calorie-restricted rats ($F = 0.2$, $p =$ NS).

The daily pattern of adrenal gland corticosterone concentration tended to mirror that of blood (Figure 1, lower panel). When analyzed as a main factor in a factorial ANOVA, calorie restriction significantly reduced ($p < 0.00001$) the 24 h adrenal gland corticosterone level ($F_{1,78} = 20.6$). Time-of-day changes were significant in calorie-restricted animals only, with a maximum at the beginning of the rest span of the daily photoperiod (one-way ANOVA).

As shown in Figure 2 (top), calorie restriction significantly decreased plasma GH levels ($F_{1,78} = 37.8$, $p < 0.00001$) and distorted its 24 h pattern ($F_{5,78} = 10.4$, $p < 0.0001$ for the interaction "time × diet" in the factorial ANOVA). In control rats, the pattern of GH secretion was defined by peak values during the first part of the rest span (1 to 5 HALO) followed by decreases to a plateau that lasted throughout the entire activity span. Calorie-restricted rats exhibited very low circulating GH concentrations at all the examined times of day.

Figure 2 (bottom) presents the 24 h pattern of plasma leptin. A factorial ANOVA indicated significant effects of treatment and time of day ($F_{1,78} = 224.6$, $p < 0.00001$ and $F_{5,78} = 2.82$, $p < 0.02$) as well as a significant interaction "treatment × time" ($F_{5,78} = 4.25$, $p < 0.003$), *i.e.*, calorie-restriction decreased plasma leptin and changed its 24 h secretory pattern. The 24 h pattern of leptin in control rats was characterized by a maximum and a minimum at the middle of the activity and resting phase, respectively. Calorie-restriction caused a reversal of the pattern, with a maximum in plasma leptin concentration in the middle of the resting phase (Figure 2, bottom).

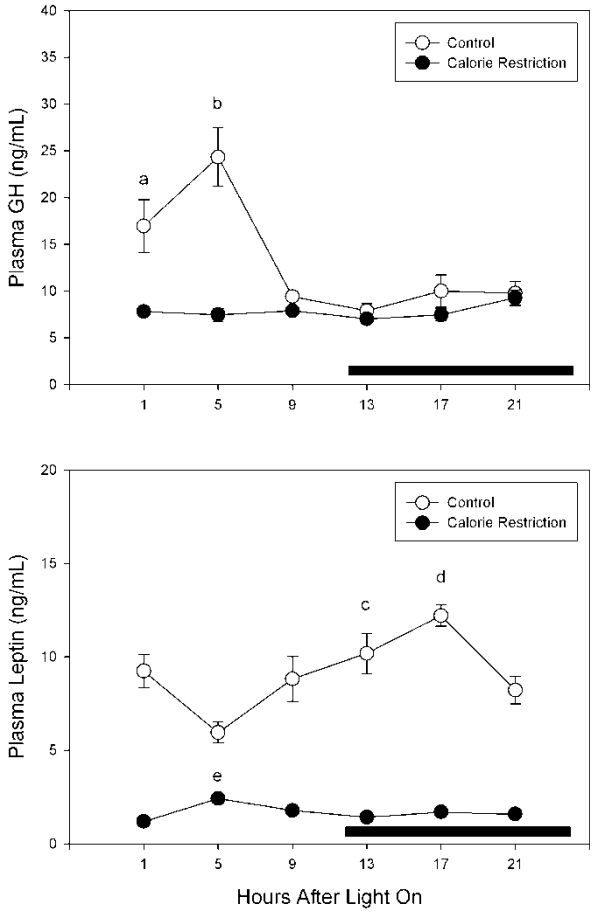

FIGURE 2 Effect of calorie restriction on 24 h changes in plasma GH (top) and leptin levels (bottom) in male rats. Groups of 7 to 8 rats were killed by decapitation at 6 different times throughout a single 24 h cycle. Bar indicates scotophase duration. Results are the means ± SEM. Letters indicate the existence of significant differences between the time points means within each group by one-way ANOVA followed by Tukey-Kramer's multiple comparisons test, as follows: [a]$p < 0.05$ versus 13 HALO; [b]$p < 0.01$ versus 9, 13, 17, and 21 HALO; [c]$p < 0.05$ versus 5 HALO; [d]$p < 0.05$ versus 21 HALO, $p < 0.01$ versus 5 HALO; [e]$p < 0.05$ versus 1, 13, 17, and 21 HALO. For further statistical analysis, see text.

The results of the second study designed to assess changes in plasma ACTH and corticosterone concentration in group-caged rats, isolated control rats, and calorie-restricted rats during the rest span of daily photoperiod are depicted in Figure 3. With reference to plasma ACTH (Figure 3, top), a factorial ANOVA indicated a significant effect of experimental manipulation and time of day ($F_{2,47} = 12.1$ and 8.81, $p < 0.001$, respectively). By the Tukey post-hoc test, ACTH values in restriction conditions were found to be significantly lower than those of both the isolated and group-caged controls ($p < 0.01$), and the values at 9 HALO

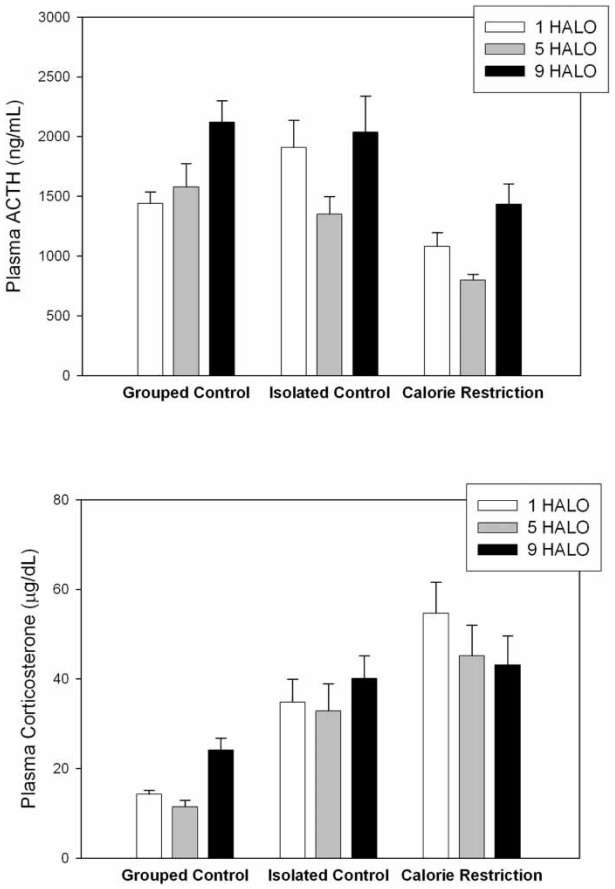

FIGURE 3 Plasma ACTH (top) and corticosterone concentration (bottom) in group-caged and isolated rats, and calorie-restricted rats during the rest span of daily photoperiod. Shown are the means ± SEM (7 to 8 rats per group). A factorial ANOVA followed by a Tukey post-hoc test indicated that ACTH values in the calorie-restriction conditions were significantly lower than those of the isolated and group-caged controls ($p < 0.01$) and that ACTH values at 9 HALO were significantly higher than those at 1 HALO ($p < 0.04$) or 5 HALO ($p < 0.0001$). In the case of plasma corticosterone, group-caged control showed the lowest corticosterone concentration ($p < 0.001$, post hoc Tukey test). Plasma corticosterone levels of calorie-restricted rats were significantly greater than those of controls ($p < 0.03$). For further statistical analysis, see the text.

were significantly higher than those at 1 HALO ($p < 0.04$) or 13 HALO ($p < 0.0001$). In the case of plasma corticosterone (Figure 3, bottom), only a significant effect of experimental manipulation was found by factorial ANOVA ($F_{2,47} = 24.2$, $p < 0.0001$), the group-caged controls showing the lowest corticosterone concentration ($p < 0.001$, post hoc Tukey test). The plasma corticosterone levels of the calorie-restricted rats during the rest span of the daily cycle were significantly greater than those of the controls ($p < 0.03$).

DISCUSSION

Temporal organization is an important feature of the biological systems, and its main function is to facilitate adaptation to the environment (Moore-Ede, 1986). The daily variation of biological variables arises from an internal time-keeping system, and the major action of the environment is to synchronize this internal clock to a period of exactly 24 h. The light-dark cycle, food, ambient temperature, scents, and social cues have been identified as environmental synchronizers or "Zeitgebers" in rats (Hastings, 2003; Moore-Ede, 1986). Food availability acts as a "Zeitgeber" resulting in a "food anticipatory activity" (Mistlberger, 1994) that occurs only if feeding intervals are within the circadian range (Stephan, 1981). Circadian rhythms exhibit a gradual resetting in response to mealtime shifts (Stephan, 1984; Stephan, 1992) and free-run during total food deprivation (Coleman et al., 1982; Rosenwasser et al., 1984; Stephan, 1984; Stephan, 1992).

A number of studies have indicated that a hyperadrenocortical state is found in calorie-restricted rats (Sabatino et al., 1991). Apparently, this does not involve an activation of the hypothalamic-pituitary axis, because ACTH levels were not found to be elevated in food-restricted rats (Han et al., 1995). It has since been demonstrated that the basis for the hyperadrenocorticism in calorie-restricted rats resides in the adrenal cortex as the consequence of an enhanced sensitivity of adrenal cells to ACTH (Han et al., 1998). In our study, although plasma levels of ACTH decreased after calorie restriction, the actual output of corticosterone increased, mainly when assessed during the light phase of daily cycle. It is of interest to note that food-restricted rats exhibited increased free plasma corticosterone levels, mainly during the rest span of daily cycle (Nelson, 1994; Sabatino et al., 1991).

In the present study, the significant correlation between plasma ACTH and corticosterone found in control rats was no longer seen in calorie-restricted rats. Several possible interpretations may explain these results. For example, calorie restriction could affect adrenal cells directly, either through circulating catecholamines or via autonomic innervation of the adrenal gland. Alternatively, because of the hypoleptinemia developed under calorie restriction, the physiological inhibitory effect of leptin on ACTH-driven glucocorticoid secretion could be severely-abrogated, thus resulting in a ceiling of adrenal glucocorticoid output, even in the presence of very low circulating levels of ACTH (Pralong et al., 1998).

Since cannibalism commonly occurs among calorie-restricted rats, animals must be singly caged in both calorie-restricted and control groups. This added a superimposed stressful situation that was uncovered when we compared the circulating corticosterone concentration of a third

control, group-caged controls. In these animals, the circulating corticosterone levels during the light phase of the daily photoperiod were about half those of the individually housed controls and about one-third those of the calorie-restricted rats. Our results are consistent with the hypothesis that individual housing constitutes a mild stress for rodents, as shown by their increased aggression (Baurmel et al., 1978; Brain, 1975; Hurst et al., 1999) and augmented response to stressors (Giralt and Armario, 1989) reported in rats following even relatively brief periods of individual housing. The possibility should be also considered that the hormone changes seen in the isolated controls were due to the strong anticipatory and feeding activity of the nearby calorie-restricted animals.

Rats that have been calorie restricted for short periods of time exhibit altered metabolic and hormonal characteristics that persist in many cases into old age. For example, blood glucose, insulin, and triiodothyronine levels are lowered by food restriction and remained reduced throughout the life span (Herlihy et al., 1990; Masoro et al., 1992). Similarly, at an early age, food restriction reduces plasma GH levels in rats (Armario et al., 1987; Brogan et al., 1997; Bronson and Heideman, 1990). In the present study, calorie restriction brought about a significant decrease of plasma GH and a disruption of its 24 h secretory pattern.

Leptin, primarily produced by adipocytes, relays critical information to the brain regarding the current status of energy availability (Ahima and Osei, 2004; Campfield et al., 1995; Caprio et al., 2001). Leptin plays a critical role in the defense against life-threatening loss of lean body weight in times of food scarcity. Plasma leptin concentration falls in response to calorie restriction, which contributes to the resetting of neuroendocrine and behavioral systems meant to restore energy balance (Ahima et al., 1996; Maffei et al., 1995). Such an effect is clearly seen in the present study. Plasma leptin levels over the 24 h period were decreased in calorie-restricted rats, and the secretory pattern of this hormone was also altered in this experimental situation characterized by a diet low in carbohydrate and fat but high in protein. It should be noted that calorie-restricted rats exhibited the expected increase in circulating leptin levels some hours after commencing food consumption (at 5 HALO) (Kalra et al., 1999).

The changes in the secretory pattern of hormones reported here for the described experiments may be part of the neuroendocrine and metabolic mechanisms evolved to maximize survival during periods of food shortage (Holliday, 1989). Under such a circumstance, wild animals suspend growth and reproduction, induce defense molecules such as glucocorticoids, and shift whole-body fuel utilization from both carbohydrate and fat to almost exclusively fat. The effect of calorie restriction observed in this work might derive from these adaptive responses (Holliday, 1989; Masoro and Austad, 1996).

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