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# Hormonal and metabolic adaptation to fasting: Effects on the hypothalamic–pituitary–ovarian axis and reproductive performance of rabbit does

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**Abstract**

To assess the impact of acute caloric shortage on reproduction, rabbit does were either fed ad libitum (control, AL), or fasted for 24 (STF) or 48 h (LTF) before induction of ovulation with GnRH injection. Blood samples were collected during the last 3 h of fasting, and the following 4 h after GnRH injection, when feed was provided again, to measure plasma concentrations of LH, estradiol-17β, leptin, insulin, T3, corticosterone, glucose, and NEFA.

Before re-feeding, plasma leptin, insulin, and T3 concentrations were lower ($P \leq 0.01$) in both fasted groups than in controls, but then gradually increased following realimentation to match those of controls. During fasting, corticosterone levels were higher ($P \leq 0.01$) in LTF than in STF and AL does, but decreased to control values soon after realimentation. During fasting, plasma glucose concentrations did not differ among groups, but upon re-feeding they markedly increased ($P \leq 0.01$)

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both in STF and LTF does. NEFA levels were also more elevated ($P \leq 0.01$) in fasted rabbits than in controls, and rapidly decreased ($P \leq 0.01$) after re-feeding. Following GnRH injection, LH peak was lower ($P \leq 0.01$) in LTF than in AL and STF does. Estradiol-17β showed higher pulse frequency and amplitude in AL than in STF and LTF does. Compared to controls, receptivity rate of STF and LTF artificially inseminated does declined respectively by −20.5% ($P \leq 0.05$) and −22.7%, and fertility rate by −23.9% ($P \leq 0.05$) and 21.4%, but no difference was found in ovulation rate. In summary, nutritional status of does, as modified by fasting, greatly influenced fertility, metabolic and reproductive hormones.


*Keywords:* Fasting; LH; Estradiol-17β; Leptin; Insulin; T3; Corticosterone; Rabbit

## 1. Introduction

Nutrition is one of the major factors affecting the reproductive efficiency particularly in female mammals. Negative energy balance in young rabbit does can result in infertility and high culling rate because of the high energy demands for pregnancy and lactation [1].

Prolonged caloric restriction can inhibit pulsatile LH secretion by depressing the GnRH pulse generator within the hypothalamus in primates [2] and induce a condition of anoestrous in cattle [3]. Interestingly, however, even mild reduction in the level of nutrition can markedly affect reproduction [4]. Using different animal models, it was demonstrated that calories are important in the regulation of LH secretion. In primates and rats, for example, a single day of fasting is sufficient to reduce the pulsatile LH secretion pattern [5,6].

The effect of chronic fed restriction on the regulatory mechanisms of metabolism has been investigated in several animal species, but only recently in rabbits. In this species, long-term nutrient deficiency during development has major neuroendocrine consequences evoking prominent homeostatic reactions of the corticotropic, leptinergic, and thyrotropic pathways [7], but its involvement with the gonadal axis is still unclear [8]. Several metabolites, including glucose and non-esterified fatty acids (NEFA), and hormones, such as insulin and IGF-I, regulate ovulation rate, follicle development and embryo survival [4,9,10]. Recently, also leptin has been implicated in several key points of the mammalian reproductive functions [11,12]. The recent findings of leptin receptors in different ovarian structures [13] and the oviduct [14] of rabbits suggest that leptin may have a role in the steroidogenesis of pre- and post-ovulatory follicles as well as in early developmental stages of blastocyst. Leptin may act as a critical link between adipose tissue and the neuroendocrine axis, indicating whether adequate energy reserves are present for normal reproductive function [15].

Although the causal link between nutrition and fertility has been known for a long time, the underlying mechanisms by which caloric shortage affects reproductive function remain unclear and difficult to disclose [16]. Thus, complete deprivation of food for a short period of time could be a useful model for analyzing the adaptive responses of metabolic processes and their regulatory systems as well as the interrelationships between nutritional deficiency and reproductive function. The objective of the present study was to examine the effects triggered by 1- or 2-days of fasting on (1) plasma concentrations of leptin, insulin, T3, corticosterone, glucose, and NEFA as indicators of hormonal and metabolic adaptations to

food deprivation, (2) hormonal profiles of LH and estradiol-17β, as markers of the responses of the gonadal axis, and (3) reproductive performance of rabbit does.

## 2. Materials and methods

### *2.1. Animals and experimental design*

Animals were caged individually in indoor brick shed facilities and kept under a temperature of 20–28 °C by forced heating systems and a constant photoperiod of 16 h light and 8 h darkness. All animals were fed a standard commercial pellet diet ad libitum once per day, containing 11.0 MJ DE/kg dry matter and 18.3% crude protein, with free access to water. Free suckling of litters equalized to 8–9 young within 2 days after parturition and weaning at 31 days were standard procedure.

Sexual receptivity was checked on the day of insemination by evaluating vulva color. Accordingly, does were scored either receptive when their vulva was red (or purple) and turgid, or non-receptive in all the other cases. Ovulation was induced by 0.8 μg GnRH analogue (Receptal, Intervet, Milan, Italy) intramuscular injection. Artificial insemination (AI) was performed with heterospermic pool of fresh semen, assessed for mass motility under a microscope (200×) and diluted 1:5 in a commercial extender (Cortalap, IMV Technologies Italia, Piacenza, Italy), to ensure a minimum concentration of about 50 million spermatozoa per ml. The does were inseminated in the lordosis position with 0.6 ml of diluted semen through a single-use plastic pipette, following GnRH injection.

### *2.2. Experimental design*

To verify the effect of fasting on hormonal and metabolic adaptation, as well as on overall reproductive performance and the hypothalamus–pituitary–ovarian (HPO) axis, two experimental protocols were designed.

#### *2.2.1. Experiment 1*

To avoid potentially confounding effects of lactations and doe–litter interactions, only young unmated rabbit females were employed. New Zealand White (NZW, HY/CR strain) rabbits (Charles River Italia, Lecco, Italy) of 5 months age, weighing 3.5–3.8 kg were randomly assigned to one of the following three groups (12 animals/group): control (AL), short-term fasting (STF), and long-term fasting (LTF). Control does were fed ad libitum, STF and LTF does were deprived of food for 24 and 48 h, respectively, before GnRH injection. Immediately after GnRH administration (time 0), fasted does were re-fed ad libitum the same standard diet as the controls. The daily ration of feed was given to the rabbits at 8:00 a.m.

On the day of the experiment, a catheter was inserted into the central ear vein of each rabbit. Blood samples (1 ml) were collected from unrestrained catheterized animals, free to move in their cages without interfering with their normal activity, every 15 min during the last 3 h of fasting and the following 4 h after re-feeding. The samples, drawn into tubes containing EDTA, were immediately centrifuged at 3000 × *g* for 15 min and plasma was

stored frozen until assayed for hormones and metabolites. Packed cell volume was analyzed every 2 h to exclude rabbits with anemia from the experiment.

#### 2.2.2. Experiment 2

In three successive trials New Zealand White rabbit does with same parity and similar body weight were homogeneously assigned to control or treated groups. In the first trial, lactating does were either fed ad libitum (AL, $n = 38$) or fasted for 24 h (STF, $n = 43$) 1 day before AI at day 11 postpartum. In the second, non-lactating does were fed ad libitum (AL, $n = 19$) or deprived of food for 48 h (LTF, $n = 16$) before AI. The latter approach was adopted in order to avoid undue stress to young rabbits as a consequence of the drop in milk production by their foster mothers deprived of food for two consecutive days. Both STF and LTF does were re-fed ad libitum 2 h prior to AI. For each doe, the following data were recorded: body weight and feed intake the day prior to and after fasting, body weight 10 days after re-feeding, sexual receptivity at AI, fertility rate, number of total born and born-alive at parturition.

In the third trial, non-lactating rabbit does, randomly allocated ($n = 6$/group) either to control or to STF and LTF groups, were sacrificed 48 h after AI to determine the ovulation rate by counting the number of newly formed corpora lutea (CL) present in each ovary.

### 2.3. Hormone assays

Plasma concentrations of LH were determined by a homologous double antibody RIA method. The antibody used was an anti-Rb LH (AFP-3120489GP) supplied by A.F. Parlow (Harbour UCLA Medical Centre, CA) at a working dilution of 1:75,000. The tracer was obtained by iodination of rabbit LH (AFP-7818C) by the iodogen method [17]. The sensitivity of the assay was 0.5 ng/ml plasma. The intra-assay (10 determinations) and inter-assay (10 assays) coefficients of variation were 3.9 and 4.5%, respectively.

Estradiol-17β concentrations in plasma samples were assayed using a commercial $^{125}$I RIA kit (ICN Pharmaceuticals Inc., Diagnostic Division, Costa Mesa, CA, USA). The limit of detection was 0.8 pg/ml and the intra- and inter-assay coefficients of variation were <6 and <10%, respectively.

Insulin, leptin, total triiodothyronine (T3), and corticosterone concentrations were determined by RIA, as reported elsewhere [7]. Plasma insulin was determined by the double antibody/PEG technique using a porcine insulin RIA kit (Linco Research Inc., St. Charles, MO, USA). The antiserum was guinea pig anti-porcine insulin, while both labeled antigen and standards used purified recombinant human insulin. The limit of sensitivity was 2 μU/ml and intra- and inter-assay coefficients of variations were 6.8 and 9.2%, respectively. Leptin concentrations were measured by double antibody RIA using the multi-species leptin kit (Linco Research Inc., St. Charles, MO, USA). The intra-assay coefficient of variation was 3.9%. Total triiodothyronine (T3) was assayed by RIA according to the procedure provided by the manufacturer (Immunotech, Marseille, France). The sensitivity of the assay was 0.13 ng/ml, and the major analogs of T3 did not interfere with the assay. Intra- and inter-assay coefficients of variations were 4.9 and 6.1%, respectively. Plasma corticosterone concentrations were evaluated by RIA, using the CORT kit (ICN Biomedicals Inc., Costa

Mesa, CA, USA), with a sensitivity of 0.15 ng/ml and coefficient of variations of 7.6 and 9.2% for intra- and inter-assay, respectively.

### 2.4. Metabolite assays

Glucose was analyzed by the glucose oxidase method using the Glucose Infinity kit obtained from Sigma (Sigma Diagnostic Inc., St. Louis, MO, USA). The non-esterified fatty acids (NEFA) concentrations were analyzed using a two-reaction, enzymatic-based colorimetric assay from Wako (NEFA-C, Wako Chemicals GmbH, Neuss, Germany), based on the ability of NEFA to acylate coenzyme A in the presence of CoA synthetase.

### 2.5. Statistical analysis

The estradiol-17β pulses were identified in sequential blood samples collected for 7 h from both treated and control does. The criteria for identifying a single-point pulse was a value 3 S.D. above the baseline whereas those for two- and three-points pulses were 2 and 1.5 S.D., respectively [18]. The number of peaks of estradiol-17β and mean plasma values were evaluated by ANOVA followed by Student's *t*-test.

Data relative to overall treatment effects on other hormones and metabolites during the time-course of short- and long-term fasting period were analyzed by ANOVA for repeated measurements according to a model which included treatment group, doe within group, time period, and interaction between group and time period: doe within treatment was used as the error term. Comparison between effects was performed by Student's *t*-test. A value of $P < 0.05$ was considered significant. The distribution of the does in two groups according to the magnitudes of their peak LH responses to GnRH challenge was compared using a Fisher's exact test. The differences between means relative to productive performance obtained from experiment 2 were tested by Student's *t*-test, whereas proportional data were evaluated by Chi square test. All statistical analyses were performed using Prism (GraphPad Software, Inc., San Diego, CA, USA).

## 3. Results

### 3.1. Metabolic hormonal profiles

During the last 3 h of fasting, plasma leptin concentrations were lower ($P \leq 0.01$) in both STF (Fig. 1, left A) and LTF (Fig. 1, left B) does than in control rabbits, but then gradually increased matching those of AL does within the next 1–4 h after re-feeding (Fig. 1, left A and B). The length of fasting affected leptin levels, being constantly higher ($P \leq 0.01$) in LTF than in STF rabbits, before re-feeding (Fig. 1, left C). Insulin levels were five-fold lower ($P \leq 0.01$) in both STF (Fig. 1, right A) and LTF (Fig. 1, right B) does than in controls during the last 3 h of fasting, but 1 h after feed was provided again, plasma insulin concentrations rose ($P \leq 0.01$) to reach the same levels as controls in both treated groups. Upon feeding, insulin concentrations rebounded more quickly and higher ($P \leq 0.01$) in LTF than in STF does (Fig. 1, right C).

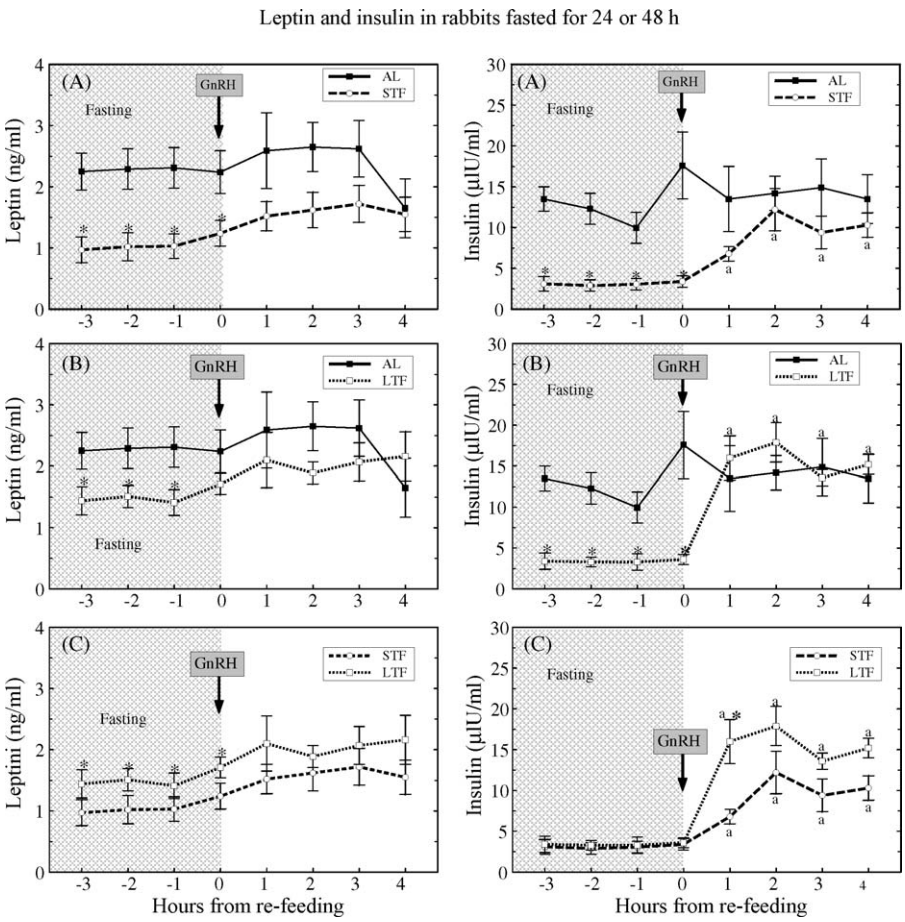


Fig. 1. Plasma leptin (left panels) and insulin (right panels) concentrations in does fed ad libitum (AL), fasted for 24 h (STF), or 48 h (LTF) before GnRH injection (arrow, time 0) and after resumption of feeding in fasted rabbits. The shaded area represents the last 3 h of fasting in STF and LTF does. Each point represents the mean ± S.E.M. of nine values. Within each panel, asterisks indicate significant ($P \leq 0.01$) different means between different experimental groups. Letters mark significant differences ($P \leq 0.01$) within the same experimental group.

During fasting, the T3 plasma concentrations were markedly ($P \leq 0.01$) reduced in both 24- (Fig. 2, left A) and 48-h fasted rabbits (Fig. 2, left B) compared to controls. Upon realimentation, T3 values rose ($P \leq 0.01$) in LTF does, whereas only a peak value ($P \leq 0.01$) was found 2 h later in STF rabbits (Fig. 2, left). By converse, in does fed ad libitum circulating T3 gradually decreased ($P \leq 0.01$) towards the end of sampling, 2 h after GnRH injection (Fig. 2, left A). During the last 3 h of fasting, the corticosterone levels were much higher ($P \leq 0.01$) in LTF does than normally fed (Fig. 2, right B) and STF (Fig. 2, right C) rabbits, but immediately after re-feeding their values dropped ($P \leq 0.01$) to match those of controls (Fig. 2, right B). By converse, no difference in corticosterone concentrations was found between STF both before and after re-feeding and control rabbits (Fig. 2, right A).

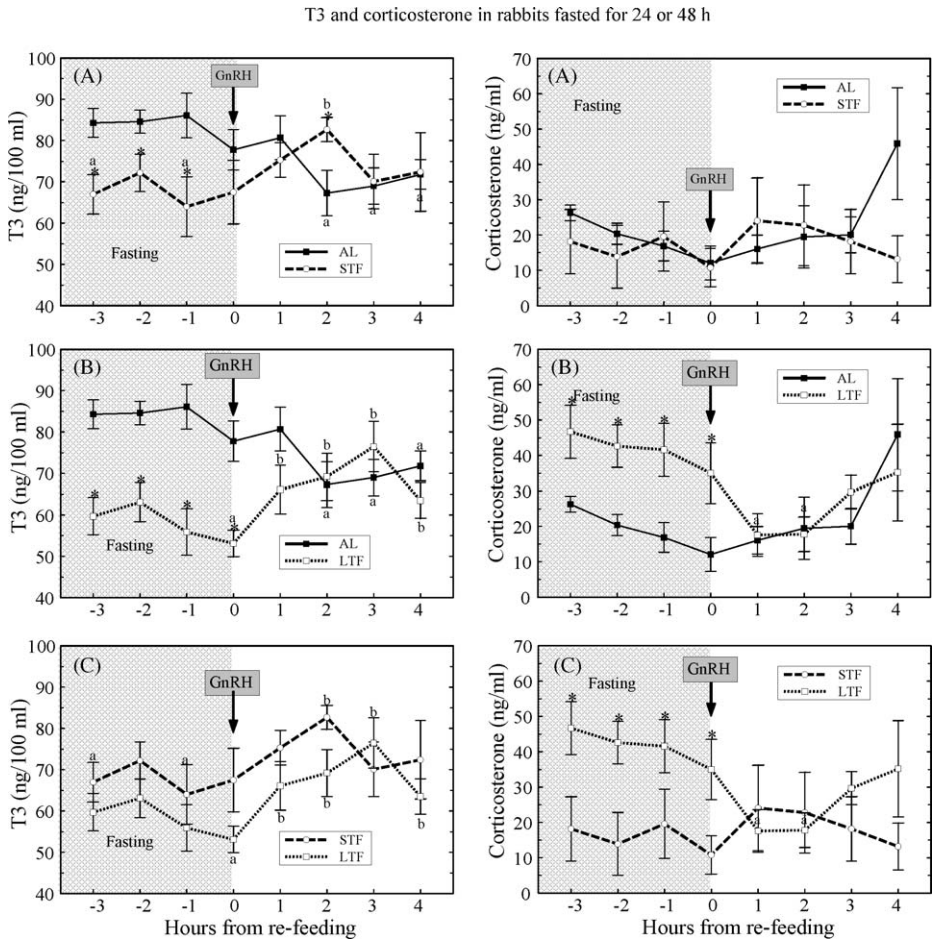


Fig. 2. Plasma T3 (left panels) and corticosterone (right panels) concentrations in does fed ad libitum (AL), fasted for 24 h (STF), or fasted for 48 h (LTF) before GnRH injection (arrow, time 0) and after realimentation in fasted animals. The shaded zone represents the last 3 h of fasting in STF and LTF does. Each point represents the mean ± S.E.M. of nine values. Within each panel, asterisks indicate significant ($P \leq 0.01$) different means between different experimental groups. Different letters mark significant differences ($P \leq 0.01$) within the same experimental group.

### 3.2. Metabolic parameters

Glucose in plasma of fully fed and 24- or 48-h fasted rabbits did not change during the last 3 h of fasting (Fig. 3, left panels). Soon after feed was provided again, glycemia rose ($P \leq 0.01$) to higher levels in STF and LTF does compared to controls during the next 4 h (Fig. 3, panels A and B). Within 1 h after feeding, glucose levels were higher ($P \leq 0.01$) in STF than in LTF does (Fig. 3, left C).

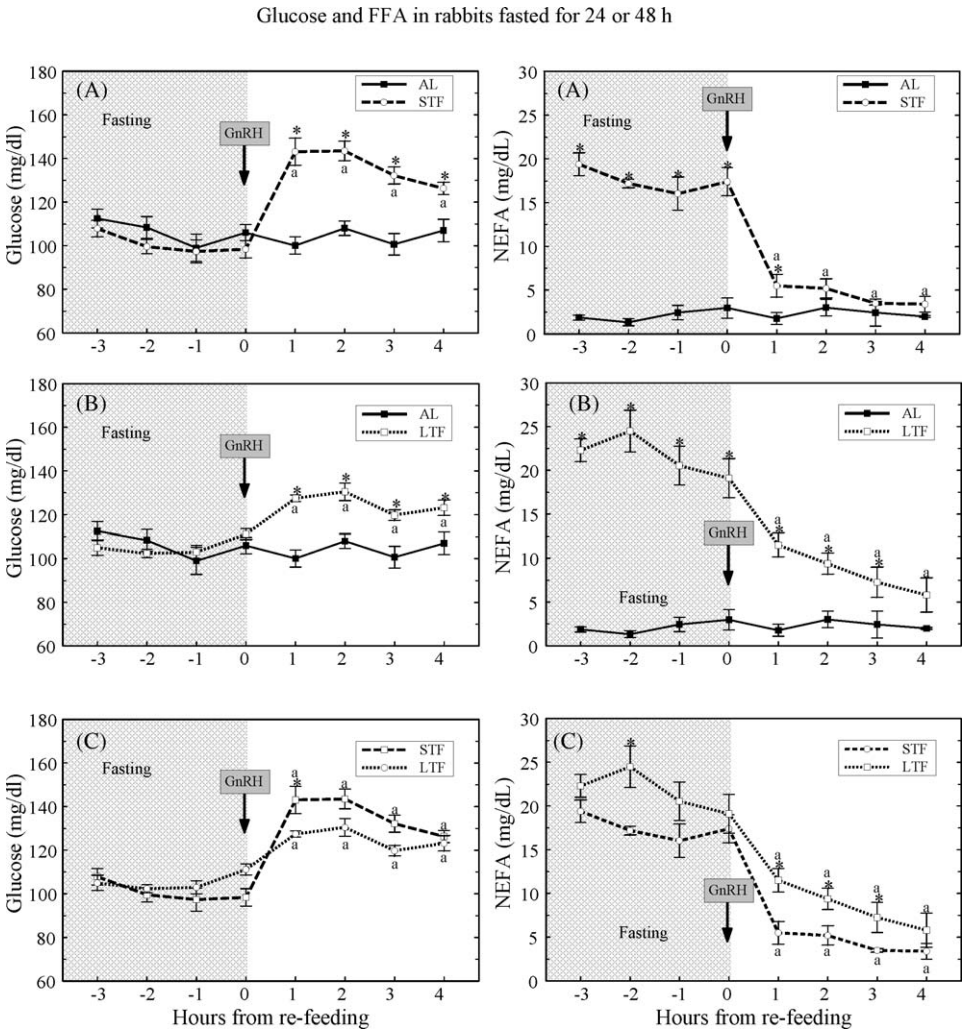


Fig. 3. Plasma glucose (left panels) and NEFA (right panels) concentrations in does fed ad libitum (AL), fasted for 24 h (STF), or fasted for 48 h (LTF) before GnRH injection (arrow, time 0) and after realimentation in fasted animals. The shaded area represents the last 3 h of fasting in STF and LTF does. Each point represents the mean ± S.E.M. of nine values. Within each panel, asterisks indicate significant ($P \leq 0.01$) different means between different experimental groups. Letters mark significant differences ($P \leq 0.01$) within the same experimental group.

During the last 3 h of complete food deprivation, both fasted groups had higher ($P \leq 0.01$) plasma NEFA concentrations than ad libitum fed rabbits (Fig. 3, right A and B). Soon after re-feeding, NEFA values rapidly declined ($P \leq 0.01$), although at different rates, in both STF (Fig. 3, right A) and LTF does (Fig. 3, right B) to values higher ($P \leq 0.01$) than controls in the first and the next 2 h, respectively. Circulating NEFA levels were

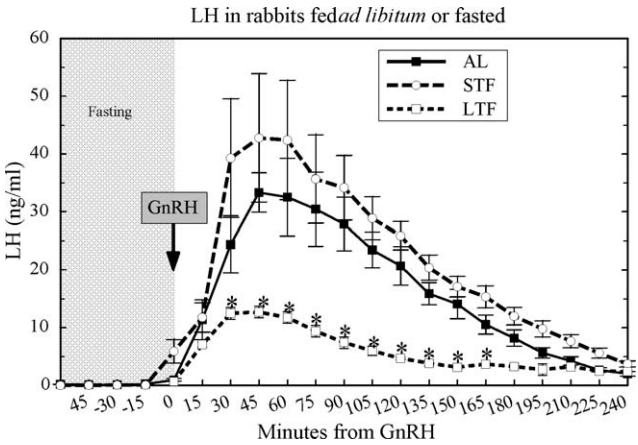


Fig. 4. Plasma LH concentrations in does fed ad libitum (AL), fasted for 24 h (STF), or fasted for 48 h (LTF) from 60 min prior to 4 h after GnRH injection (arrow, time 0) and after resumption of feeding in fasted animals. The shaded bar represents the last hour of fasting in STF and LTF does. Each point represents the mean ± S.E.M. of 12 values. An asterisk marks significant ($P \leq 0.01$) different mean value from other experimental groups.

higher ($P \leq 0.01$) in LTF than in STF does during the first 3 h after realimentation (Fig. 3, right C).

### 3.3. LH and estradiol-17β

Both short- and long-term fasting did not affect plasma LH concentrations prior to GnRH challenge which remained low and comparable to those of controls (Fig. 4). In both control and treated groups, the maximum concentrations of LH peak surge were observed 45 min after GnRH injection (Fig. 4), but their magnitudes were markedly lower ($P \leq 0.01$) in LTF does than in STF and control rabbits. In all the groups, plasma LH levels declined close to basal values by 4 h after GnRH (Fig. 4). Based on the peak value of LH secretion to exogenous GnRH, rabbits were conventionally subdivided into two groups: high (LH+, >20 ng/ml) and low (LH−, <20 ng/ml) responsive. Whereas all the 48-h fasted does were LH-, five (42%, $P < 0.01$) belonged to the STF group and only 1 (8%, $P < 0.001$) of the normally fed does, whose majority (11/12) exhibited a LH+ response.

Compared to control rabbits (Fig. 5, upper panels), estradiol-17β profiles in STF does (Fig. 5, middle panels) exhibited decreased ($2.48 \pm 0.34$ versus $4.92 \pm 0.63$; $P < 0.01$) number of pulses during this 7 h-sampling interval. In the LTF group, estradiol-17β had still longer ($0.58 \pm 0.19$ pulses/7 h; $P < 0.01$) interval between pulses than in STF does and in nine rabbits out of the 12 examined it remained constantly low (Fig. 5, lower panels). The mean level of estradiol-17β release was lower in LTF than in control does ($2.60 \pm 0.53$ pg/ml versus $10.73 \pm 3.71$ pg/ml, respectively; $P < 0.01$), and $4.30 \pm 0.97$ pg/ml in STF rabbits. Within each treatment group, there was no clear difference in the estradiol-17β patterns of release between fasting and subsequent feeding, either before or after GnRH administration (Fig. 5).

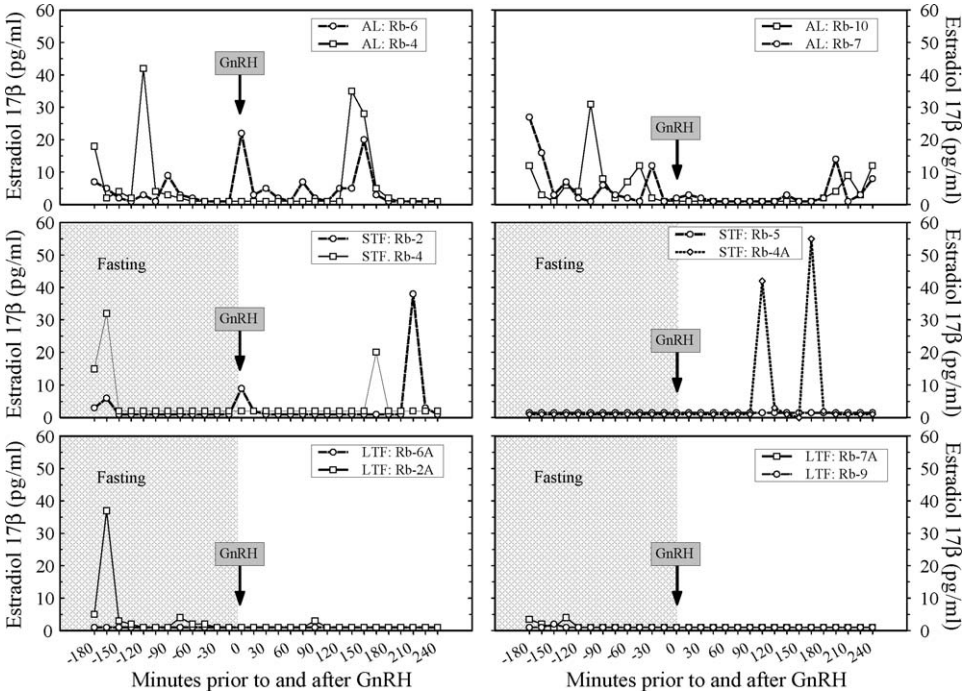


Fig. 5. Plasma levels of estradiol-17β in sequential, 15-min samples collected from four different, representative, control rabbits fed ad libitum (AL, upper panels), or 24-h (STF, middle panels) and 48-h (LTF, lower panels) fasted does during the last 3 h of fasting (shaded bars) and the following 4 h after GnRH injection (arrows, time 0). Immediately after GnRH injection, feed was provided ad libitum to previously fasted animals.

### *3.4. Reproductive performance*

The overall reproductive performances of does deprived of food for 1 or 2 days before AI are summarized in Tables 1 and 2, respectively. In lactating does (Table 1), STF reduced

Table 1
Effect of 24 h of fasting (STF) before insemination at day 11 postpartum on reproductive performance of lactating rabbit does (mean ± S.D.)

| | STF | Control |
|---|---|---|
| Does/litters (no.) | 43 | 38 |
| Receptivity (%) | 55.8 a | 76.3 b |
| Fertility (%) | 41.9 a | 65.8 b |
| Total born (no.) | 7.8 ± 4.8 | 7.5 ± 3.5 |
| Born alive (no.) | 6.9 ± 4.7 | 6.6 ± 3.6 |
| Weight variation during fasting (%) | −3.84 ± 3.0 A | 0.07 ± 3.4 B |
| Feed intake before fasting (g DM $d^{-1}$) | 376 ± 102 | 374 ± 102 |
| Feed intake after fasting (g DM $d^{-1}$) | 351 ± 142 | 313 ± 123 |

In the same row, different letters indicate significant differences between means at a, b: $P \leq 0.05$; A, B; $P \leq 0.001$.

Table 2
Effect of 48 h of fasting (LTF) before insemination on reproductive performance of non-lactating rabbit does (mean ± S.D.)

| | LTF | Control |
|---|---|---|
| Does/litters (no.) | 16 | 19 |
| Receptivity (%) | 56.2 | 78.9 |
| Fertility (%) | 31.2 | 52.6 |
| Total born (no.) | 10.2 ± 3.6 | 10.7 ± 2.7 |
| Born alive (no.) | 8.6 ± 4.1 | 10.7 ± 2.7 |
| Weight variation during fasting (%) | −7.0 ± 3.5 A | −1.7 ± 3.9 B |
| Feed intake before fasting (g DM $d^{-1}$) | 302 ± 21 | 301 ± 42 |
| Feed intake after fasting (g DM $d^{-1}$) | 297 ± 55 | 259 ± 55 |

In the same row, different letters indicate significant differences between means at A, B: $P \leq 0.001$.

($P \leq 0.05$) both receptivity (−20.5%) and fertility rate (−23.9%) in comparison with controls, but did not influence litter size and fetal viability at birth. The STF affected body weight of treated does which was almost 4% lower ($P \leq 0.001$) than that of fully fed control does in the same time interval. Interestingly, however, soon after realimentation, feed intake of treated does increased together with a gain in body weight between days 12 and 21 (3.38 ± 5.1% versus −1.40 ± 5.3%; $P \leq 0.001$) of lactation, so that they matched the weight of controls (4439 ± 532 g versus 4469 ± 493 g).

Also LTF appeared to negatively influenced sexual receptivity (56.2% versus 78.9%; $P = 0.16$), fertility rate (31.2% versus 52.6%; $P = 0.35$), and litter size at birth (8.6 versus 10.7; $P = 0.27$), but the differences were not significant due to the low number (16 versus 19) of observations (Table 2). The LTF greatly influenced body weight of treated does which, at the end of fasting, was 5.3% lower ($P \leq 0.01$) than that of control does. Within 10 d following realimentation, this loss was regained, so that both treated and control does had a similar body weight (4608 ± 332 g versus 4558 ± 234 g).

All control and fasted does ovulated following GnRH injection. Ovulation rates did not differ among controls (9.7 ± 2.1 CL), STF and LTF does (9.3 ± 2.4 and 8.6 ± 2.3, respectively).

## 4. Discussion

The effect of acute feed restriction on fertility has been investigated in several animal species [19–22], but only marginally in rabbits [1,8]. Although the basic underlying physiological responses to fasting might be common in mammals, to our knowledge this is the first report to describe the hormonal and metabolic homeostatic adaptations that occur in response to acute food deprivation and their repercussion on the neuroendocrine axis and fertility of rabbits. Moreover, complete feed restriction spanned the 2 days of fasting just before ovulation to exacerbate the impact of maternal nutritional status on reproductive function. In the present study, however, we were mainly interested in verifying the hormonal and metabolic dynamic changes during the transition from fasting to fed status, just before and after GnRH-induced ovulation, rather than throughout the whole period of fasting.

Compared to rabbits fed ad libitum, fasting altered metabolic hormones and metabolites differently. Decreased plasma concentrations of leptin, insulin, and T3 and increased NEFA concentrations were all important events for the metabolic adaptation to fasting together with the activation of the adrenal axis in more severely feed deprived rabbits. During fasting, similar modifications have been described in all the species so far studied [4,19,23–25] demonstrating that substantial homology exists in mammalian species in the regulation of energy homeostasis in coping with nutritional stress.

In the present study, fasting caused a clear reduction in plasma concentrations of both insulin and leptin, probably in response to reduced availability of carbohydrates. Similar findings were observed in several other species [23,26–30] and in young growing rabbits as a consequence of the restricted feed intake [7]. Insulin is a key player in the control of intermediary metabolism, and exerts an important role in ovarian function in many species. Since there is evidence of active transfer of both insulin and leptin into the brain, these hormones could have a role in signaling the metabolic state of the animal and in the regulation of appetite [31]. However, the mechanisms through which fasting decreases circulating leptin levels are still poorly understood. An increasing body of evidence suggests that leptin secretion is regulated by other factors not directly dependent on adipose body mass content [32,33], but rather on the availability of oxidizable nutrients [34]. Notably, there was also a differential leptin response to the severity of fasting. Unexpectedly, in fact, in LTF does leptin concentrations, although lower than in controls, were higher than those of 24-h fasted rabbits. This differential response may be partially explained by the stimulatory action of corticosterone, whose peripheral plasma concentrations were indeed much higher in LTF than in STF rabbits. In fact, dexamethasone administration was found to potently stimulate leptin mRNA expression and release in cows [35].

Changes in the energetic balance clearly affected systemic thyroid hormone concentrations in does. In fact, during fasting, T3 concentrations were lower than in control rabbits. Thus, food deprivation reduced thyroid function so that animals could spare energy by decreasing adaptive thermogenesis [36]. Similar down-regulation of thyroidal function was also found in growing rabbits during long-term feed restriction [7] and also in several other species, when undernourished [37,38]. According to recent views, the drop of thyroid hormone during starvation is mediated by decreased expression of thyrotropin-releasing hormone in the hypothalamus upon falling leptin levels [39].

Corticosterone plasma concentrations were higher in 48-h fasted rabbits compared both to ad libitum fed and 24-h fasted animals. These high corticosterone circulating concentrations may represent an adaptation to the prolonged metabolic stress induced by complete food deprivation. However, the activation of the adrenal axis may have both beneficial effects, by stimulating gluconeogenesis, and several other detrimental side effects resulting in disturbances at both hypothalamic–pituitary and ovarian levels, similarly to what was described by other authors [40]. Conversely, corticosterone was found to decrease under prolonged feed restriction in rabbits, counteracting the excessive catabolic rate of basal metabolism [7].

In both 24- and 48-h food deprived rabbits, glucose was maintained at a steady state level comparable to that of ad libitum fed rabbits. Conversely, long-term underfeeding was found to reduce glucose concentration in rabbits [7]. In our experimental model, NEFA concentrations were higher in feed restricted rabbits than in control toward the end of

fasting. Moreover, the longer the fasting the higher the NEFA concentrations. Free fatty acids are released by the action of hormone sensitive lipase on triglyceride stores in adipose tissue and increased NEFA concentrations are indicative of negative energy balance [41]. Hence, augmented circulating NEFA during fasting may reflect redirection of fat metabolism towards increased lipolysis due to decreased plasma insulin concentrations.

Interestingly, leptin, insulin, T3, and corticosterone adapted very quickly from fasting to re-feeding conditions, as their plasma concentrations were comparable to controls within a few hours after realimentation. During the immediate realimentation period, leptin levels gradually rose to match those of rabbits fed ad libitum in both fasted groups. This finding suggests, indirectly, that leptin may signal the current availability of nutrient oxidizable fuel [34] rather than the overall metabolic energy stored in fat depots in accordance with what was reported in dairy cattle [30]. Within 1 h after realimentation, insulin quickly rebounded to normal values, probably in response to increasing glucose concentrations derived from absorption of carbohydrate with food intake. Thus, when does start to feed again a sudden shift from fat to carbohydrate metabolism occurs and secretion of insulin increases. Similar findings were reported in a variety of species [3,25,30]. These acute changes in blood glucose and insulin might have mediated the leptin response of rabbits to realimentation [42]. Whereas low insulin during the fasting state facilitates lipolysis, high insulin following post-fast realimentation stimulates de novo lipogenesis [43]. In our study, T3 concentrations rose to values similar to those in ad libitum fed rabbits 1 h after re-feeding. The mechanisms by which food intake and availability of different carbon sources activate the thyroidal axis are not entirely understood, but recent studies provided experimental evidence about the role of the leptin pathway [39].

Immediately upon re-feeding, glucose concentrations rose by 30–40% over those of controls in both fasted groups of does, and remained sustained the next 4 h. The accompanying hyperinsulinemia suggests that acute fasting may induce insulin resistance. However, the possibility that this hyperglycemia depends on increased voluntary food intake triggered in previously fasted does cannot be ruled out. The increased food intake aimed at recovering the body weight lost during fasting is probably favored by low leptin and insulin concentrations, which increase anabolic central neuronal pathways [44]. Within 2 h after re-feeding, NEFA concentrations in STF does declined to values comparable to those of ad libitum fed animals in coincidence with increased insulin and glycemia. Conversely, in LTF does, the decrease of NEFA after re-feeding was more gradual, even though insulin and glucose were much higher than in STF animals.

In the present study, fasting influenced the neuroendocrine axis by modifying the response of the anterior pituitary gland to GnRH and ovarian steroidogenic activity. Acute food deprivation negatively affected the temporal release patterns of estradiol-17β, whose pulse frequency and amplitude were lower in 48-h fasted than in both control and 24-h fasted animals. The rapid decline of estradiol-17β concentrations from peak values observed in some STF does in the early post fasting period could be due to an increased estradiol-17β metabolic clearance rate by the liver, as reported in unfed cows whose liver blood flow increased three-fold, immediately after feeding, for the following 4.5 h [45]. However, the underlying factors responsible for the decreased estrogen levels are still unclear as they may affect the ovarian steroidogenic function either directly or indirectly through gonadotropin-related mechanisms and impaired folliculogenesis. The nutritionally derived

factors, in fact, may target both hypothalamus and pituitary, as well as the steroid competent growing follicles within the ovary; this may occur either directly or in association with systemic hormones [46], such as insulin [47,48], IGF-I [49], thyroid hormones [50], and local growth factors, including TGF-β and VEGF [51], acting in a paracrine/autocrine fashion to regulate steroidogenesis. The recent findings of leptin receptors in different ovarian structures of rabbits, including follicles at different stages of development [13], suggest that also leptin may regulate steroidogenesis of pre- and post-ovulatory follicles. Moreover, leptin was found to inhibit the production of estradiol-17β by granulosa cells under different in vitro conditions [52]. Nevertheless, there is compelling evidence supporting the view that shortage of metabolizable fuels and the down regulation of many nutritional mediators, due to reduced feed intake, may directly influence the steroidogenic capability of ovarian follicles through gonadotropin-independent mechanisms similarly to what was found in gilts [51,53]. Thus, the fasting-induced fall in circulating estradiol-17β was likely the combined results of a reduced synthesis by the ovary and increased clearance by the liver.

In the present study, the neuronal limb of the ovulatory reflex was bypassed by the exogenous administration of GnRH; this acted on the anterior pituitary to trigger LH release within a few minutes following binding to GnRH receptors. However, the LH peak surge was consistently low in every 2-day fasted doe and also in 42% of rabbits fasted for 24 h. It is noteworthy that the GnRH-dependent LH surge showed considerable doe-to-doe variation particularly within the STF group, but the reasons for this nutritionally dependent variability still remain unclear. In fact, it can be ascribed to individual genetic susceptibility to metabolic stress and/or to their different pre-fasting metabolic condition. It remains to be established, however, which factors were actually responsible for the fasting-induced reduction of LH secretion, as this might be due to down regulation of pituitary GnRH receptors and/or to reduced synthesis of LH by pituitary cells.

In the present study, the role of estradiol-17β in the maintenance of a functional hypothalamic–pituitary axis capable of responding appropriately to GnRH appears to be essential; in fact, STF and LTF rabbits showed a lower hormonal pulse frequency and most of the LTF does had very low plasma estrogen concentrations. However, the sites of action within this system and the mechanisms whereby decreasing estradiol-17β concentrations inhibit GnRH-induced LH surge are not yet clear. In rabbits, moreover, the reflex LH surge-generator neuronal apparatus is usually unresponsive to the positive feedback by estrogen, which is in striking contrast with spontaneous ovulatory species [54].

A direct role of leptin in the neuroendocrine regulation of energy homeostasis [55,56] and gonadotropin secretion by the pituitary [23] cannot be ruled out. However, from our data there is little evidence that leptin may act in the short term to regulate pituitary LH secretion and ovarian estradiol-17β release. In fact, in does fasted for 24 h, the pituitary responses to GnRH, as well as estradiol-17β patterns, were normal in 58% of animals, despite their having severely reduced peripheral plasma leptin concentrations. These results, rather than being the failure of leptin to directly signal caloric shortage to the neuroendocrine axis and the ovary, may reflect the maintenance of an adequate or close-to-adequate level of oxidizable metabolites in 1-day fasted rabbits. Taken together, these findings indicate that leptin regulation of the GnRH/LH secretory system may depend upon the concurrently steroid milieu and overall nutritional status. Thus, it is more plausible that leptin exerts a permissive role,

together with other metabolic hormones and different nutrients, in signaling body condition and energy reserves to the hypothalamus–pituitary–ovarian axis for controlling reproductive function.

Insulin was found to directly influence LH release by the anterior pituitary in rats [57]; at the same time, it may exert a direct effect on the ovary by enhancing steroidogenesis [48]. Consequently, the low plasma insulin levels might be responsible for the impaired estradiol-17β synthesis and release by the ovaries of fasted rabbits. However, the low insulin hypothesis per se does not fully explain the present results, given the differential gonadal and pituitary response observed between does fasted for 24 or 48 h.

In adult female rats, hypothyroidism inhibits follicular development and estrogen secretions by the ovary and lowers LH pituitary response to GnRH [50]. These findings suggest that a similar functional relationship between thyroid and both gonadotropic and gonadal hormones may exist also in the rabbit during fasting.

In the rat, there is evidence that activation of the hypothalamic-pituitary-adrenal axis is responsible for the fasting-induced suppression of LH secretion, because administration of a corticotropin-releasing hormone antagonist can reverse the hypogonadotropism arising from food deprivation [6]. Glucocorticoid receptors have been localized in rat granulosa cells and cortisol was found to inhibit FSH-stimulated aromatase. [58]. These results, together with our findings on the up regulation of the adrenal axis in 2-day fasted does, suggest that the adrenal axis may be deeply involved in the down regulation of the reproductive axis, acting either centrally or peripherally.

Independently of the duration of fasting, the present data show that caloric shortage negatively affected sexual receptivity and fertility rate, but not ovulation rate, litter size and mortality rate at birth, which is considered a good indicator of offspring health. Although both STF and LTF resulted in a temporary body weight loss in does, they subsequently recovered within a few days due to increased feed intake and compensatory growth. At present, however, it is not clear what metabolic signals are specifically involved in all these nutritionally related effects induced by acute food deprivation on reproduction, nor whether they act directly upon the hypothalamic–pituitary–ovarian axis or indirectly to regulate the secretion of other hormones, such as GH [55] or prolactin; these in turn may influence the gonads. Changes of the metabolic status due to short-term feed restriction have been found to cause a large array of negative effects on reproduction in a variety of species: delaying puberty, altering estrous cycles, inhibiting estrous behavior, and influencing both oocyte maturation and embryonic survival [4,20,59–63].

In summary, the present data evidenced changes in circulating reproductive, as well as metabolic, hormones and in key metabolites evoked by acute caloric deprivation just before ovulation. Pre-conception nutritional status of the does, as modified by fasting, greatly influenced fertility and sexual receptivity rather than the ovulatory index. Before ovulation, a constellation of hormones and oxidizable metabolites are likely involved in signaling the energy status of the rabbits to the central reproductive axis and ovary, thus influencing follicular development and oocyte quality. Taken together, these results may contribute to attain more insight into possible hormonal mechanisms mediating nutritional effects on ovulation and reproductive function. In this respect, the rabbit can represent an interesting animal model, given that, following GnRH exogenous induction, its ovulation is precisely timed.

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