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Title: Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in energy homeostasis and metabolic programming

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Abstract: This study examined the effects of food restriction during rabbit pregnancy on hormones and metabolites involved in energy homeostasis and metabolic programming. Pregnant does were assigned to four groups: the control group was fed a standard ration while the others received a restricted amount of food (30% restriction) during early (0-9 d), mid (9-18 d), and late (19-28 d) pregnancy. The pregnancy induced a coordinated range of adaptations to fulfil energy requirements of both mother and foetus, such as hyperleptinemia and hyperinsulinemia, reduced insulin sensitivity, increased cortisol and non-esterified fatty acid. Food restriction altered leptin, insulin, T3, non-esterified fatty acids and glucose concentrations depending on the gestational phase in which it was applied. Collectively, present data confirm that the endocrinology of pregnancy and the adaptive responses to energy deficit makes the rabbit an ideal model for studying nutritional-related disorders and foetal programming of metabolic disease.

Suggested Reviewers:

Revision Note

Title: Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in energy homeostasis and metabolic programming
Authors: L. Menchetti, G. Brecchia, C. Canali, R. Cardinali, A. Polisca, M. Zerani, C. Boiti
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To the Editor-in-Chief
Research in Veterinary Science

We thank the referees for providing constructive comments and help in improving contents of this paper. Please note that the paper has been re-formulated and the title changed accordingly to Referee 1. Furthermore, the discussion has been reduced by eliminating non-essential information. We've also replaced the table with line graph figures. Additionally, we've included all the corrections suggested by Referee 2 in the pdf file. Below we provide answers to the reviewers' comments.

Reviewer #1*: The study "Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in metabolic programming" offers very interesting data on the effects of nutritional restriction on the metabolic parameters of pregnant does. These data are of interest and, fitting well with the scope of the journal, deserves publication. However, the authors give the false idea that they are studying metabolic programming. Metabolic programming is a very complex trait and there is no evidence to be occurring in the animas studies by the authors. There is no information on any gene regulating metabolic programming or in any change of the pre- and postnatal phenotype of the offspring that may assure that they are being programmed. Hence, if the authors do not have such data, I think that the manuscript must be rewritten in agreement; a little sentence about possible involvement in the metabolic programming of the parameters in the introduction section and a little paragraph in discussion is more than enough.*

Response. We agree with the reviewer that there is no direct evidence to support metabolic programming in our animals. Thus, we have shifted the focus of the paper from metabolic programming to energy metabolism. We have changed the title (from "*Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in metabolic programming*" to "*Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in energy homeostasis and metabolic programming*") and the introduction (it has been fully rewritten). The new version of introduction and discussion sections contains only few references to metabolic programming in order to justify the choice of the parameters. We agree with the referee that further investigations of offspring phenotype should be done, especially using the rabbit as animal model, and we hope our data will contribute to future endeavors in this regard.

Reviewer #2: *The manuscript could benefit from some additional copy reading, but overall the preparation is adequate. The data and the statistical analyses of the data are very interesting, but the conclusions drawn from the data in the manuscript are often not*

warranted or are overarching and speculative. Quite a lot of the material in the Discussion could be eliminated. Placing all of the data in a single table makes it laborious to connect the written description of the data with the data itself. Most of the data would be better presented as line graph figures with each line representing a different treatment group, the concentration of the analyte on the y axis and day of gestation on the x axis. This type of presentation of the data would also make the time component stand out better. Please see the attached pdf file of the corrected manuscript for detailed corrections. I would definitely like to see a revised copy before proceeding with publication.

Response. We have rephrased some conclusions thanks to Referee comments listed in the pdf file. In particular, we have toned down the claims about insulin (see Page 9, Line 211), and cortisol (speculative conclusion in abstract has been deleted and references have been added at Page 10, Line 232). Moreover, conclusion reaching beyond the scope of the leptin data have been deleted.
As suggested by the referee, we have included more details about ANOVA and post-hoc analysis of insulin (see Page 6-7, Lines 133-6) and T3 (see Page 7, Lines 140-5).
Furthermore, the discussion has been reduced by eliminating non-essential parts.
As suggested by the referee, we have reported in graph figures many of the results previously presented in the table (leptin and insulin as Figure 1A-1B, T3 and cortisol as Figure 2A-2B, NEFA and glucose as Figure 3A-3B) and we've left HOMA-IR as Table (Table 2).
Additionally, we've included all the corrections suggested by Reviewer in the pdf file.

Specific comments
Comment 1, Pag 1 and 2, Li 25: *spell out.*
Response: as suggested, we have spelled out "NEFA".

Comment 3-4, Pag. 2, Li 26-28: *the….diet*
Response: this sentence has been deleted.

Comment 5, Pag 1, Li 26-28: *I would not make this conclusion- rephrase the interpretation of the cortisol data*
Response: we have toned down the claims about cortisol and this sentence has been deleted.

Comment 6, Pag. 2, Li 30: *…being*
Response: we have corrected this throughout the paper.

Comment 7-8, Pag 3, Li 42: *…are….the*
Response: this sentence has been eliminated.

Comment 9, Pag 3, Li 55: *rodents undergo a lot of postnatal brain development pre-weaning. Please rephrase this statement*
Response: we have completely reworked and rewritten the introduction.

Comment 10, Pag. 4, Li 66 and 72: *spell out*

Response: the introduction has been fully rewritten. In this new version we have checked acronyms.

Comment 11, Pag. 4: ~~*Thus*~~
Response: as suggested, we have eliminated this word.

Comment 12-13, Pag 5-6: *spell*
Response: we have checked acronyms.

Comment 14, Pag. 6, Li 115: *inter-assay*
Response: as suggested, we have corrected this error.

Comment 15, Pag 6, Li 123: ~~*obtained*~~
Response: we have corrected this.

Comment 16, Pag 7, Li 137: ~~*P,*~~ *p*
Response: we have corrected "*P*" with "*p*" throughout the entire text.

Comment 17, Pag 7, Li 146: *values were*
Response: we have changed this sentence according to referee.

Comment 18, Pag 7, Li148: *(0-26)?*
Response: we have stated that, as rightly suggested.

Comment 19, Pag 7, Li 148: *groups*
Response: this word has been added.

Comment 20, Pag 7, Li 148-150: *groups was <u>not</u> significant for insulin concentration.*
Response: we have included more details about ANOVA and post-hoc analysis of insulin (see Li 134-137).

Comment 21, Pag. 7, Li 154-155: *this was true for at least one value and the mean for R1 – R2, but not for R3*
Response: we thank the referee to alerting us of this; we have rephrased this sentence (see Li 143-146).

Comment 22, Pag 7, Li 155-156: *concentrations…..consistently*
Response: these suggested edits have been made.

Comment 23, Pag 8, Li 170-172: *modified this pattern…..during…*
Response: both of these suggested edits have been made.

Comment 24, Pag. 8, Li 176 and 178: ~~*By converse*~~*…* ~~*that modified*~~
Response: we have eliminated these words.

Comment 25, Pag. 8, Li 186: *concentration*
Response: we have corrected this word.

Comment 26, Pag. 9, Li 192-197: *conclusion reaches beyond the scope of the data*
Response: we agree with the referee and we have eliminated this.

Comment 27-30, Pag. 9, Li 201, 202, 203, and 207: ~~*Thus,*~~ *…may… and… consistent with*
Response: these suggested edits have been made.

Comment 31, Pag. 9, Li 213: *concentration*
Response: we have corrected this.

Comment 32-33, Pag. 10, Li 217 and 223: *in… the*
Response: both of these suggested edits have been made.

Comment 34, Pag. 10, Li 225-228: *only GxT was significant for insulin. The trend is there, but the conclusion reached is too strong for the data*
Response: we have toned down the claims about insulin (see Li 211-215) and we reported results of post-hoc analysis (Bonferroni's test).

Comment 35, Pag. 10, Li 240: ~~*clearly*~~
Response: we have deleted the word.

Comment 36, Pag. 11, Li 246: *citation*
Response: we have added bibliography about changes of hypothalamic-pituitary-adrenal axis during pregnancy: a review published in *Journal of Neuroendocrinology*, a symposium report in *The Journal of Physiology*, and a research paper published in *Stress.*

Comment 37, Pag. 11, Li 247-248: *to what (human/animal), when?*
Response: some information have been added (see Li 234-236).

Comment 38, Pag. 12, Li 270: *small*
Response: we've corrected this.

Comment 39, Pag. 12, Li 275: *in the control group*
Response: we thank the referee for this suggestion.

Comment 40, Pag. 12, Li 277-281: *not relevant*
Response: we agree with the referee and we have eliminated this.

Comment 41-43, Pag. 12, Li 282 and 283: ~~*In fact*~~*.. dietary restriction…it*
Response: these suggested edits have been made.

Comment 44, Pag. 12, Li 289-292: *not relevant*
Response: we agree with the referee and we have eliminated this sentence.

Comment 45-46, Pag. 12, Li 292: ~~*taken toghether*~~*…..may be associated with*
Response: we've included these corrections.

Comment 47-48, Pag. 13, Li 293-294: *difference in…in each phase of gestation*
Response: both of these suggested edits have been made.

Comment 49-52, Pag. 13, Li 297-303: *associations with…that could be studied in this animal model…. in people, in other species?*

Response: we thought that this phrase is redundant and, according to Reviewer 1, we have deleted it.

Comment 52-54, Pag. 13, Li 306, 307-308: *some…~~thus~~…work*
Response: these suggested edits have been made.

Comment 55, Pag. 22: *should be "Table 2"*
Response: we thank the referee to alerting us of this; we have corrected this typo.

Comment 56, Pag. 21: *use line graphs to better illustrate the time component-leave HOMA-IR as table*
Response: we understand the confusion given by the use of the single table. As suggested by the referee, we have replaced it with line graph figures (leptin and insulin as Figure 1A-1B, T3 and cortisol as Figure 2A-2B, NEFA and glucose as Figure 3A-3B) and we've left HOMA-IR as Table (Table 2).

Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in energy homeostasis and metabolic programming

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Abstract

This study examined the effects of food restriction during rabbit pregnancy on hormones and metabolites involved in energy homeostasis and metabolic programming. Pregnant does were assigned to four groups: the control group was fed a standard ration while the others received a restricted amount of food (30% restriction) during early (0-9 d), mid (9-18 d), and late (19-28 d) pregnancy. The pregnancy induced a coordinated range of adaptations to fulfil energy requirements of both mother and foetus, such as hyperleptinemia and hyperinsulinemia, reduced insulin sensitivity, increased cortisol and non-esterified fatty acid. Food restriction altered leptin, insulin, T3, non-esterified fatty acids and glucose concentrations depending on the gestational phase in which it was applied. Collectively, present data confirm that the endocrinology of pregnancy and the adaptive responses to energy deficit makes the rabbit an ideal model for studying nutritional-related disorders and foetal programming of metabolic disease.

Key words: pregnancy; rabbit; leptin; insulin; cortisol, T3, glucose

1. Introduction

In adult mammals, energy homeostasis is ensured by the integrated action of several hormones and metabolites, but during pregnancy the metabolism is reprogrammed to supply nutrients for growing foetuses and to store body reserves for lactation (Woods et al., 1998; Ladyman, 2008). These coordinate neuroendocrine events and their timing are critical not only for maintenance of normal pregnancy, parturition, and subsequent lactation but also for health outcomes later in life of newborn (Mastorakos and Ilias, 2003; Fortun-Lamothe, 2006; Augustine et al. 2008).

Several experimental models, using nutritional restriction in rats and mice, have been adopted to study the mechanisms involved in the foetal programming of adult disease (Vuguin et al., 2007; Warner and Ozanne, 2010). Previous studies on rabbits have evaluated the effects of restricted feeding during pregnancy on productive performance (Rommers et al., 2004a; Manal et al., 2010), as well as on embryo-foetal and placental development (Cappon et al., 2005; Matsuoka et al., 2012), but little is known on hormones and metabolites involved in energy homeostasis.

Rabbits have proven to be an important translational model for the study of cardiovascular and metabolic diseases due to their large size and physiologic similarities to humans (Polisca et al., 2010; Georgiev et al., 2011). In addition, the hemochorial placentation and cellular organization close to the human placenta as well as the detailed information on embryo development and embryo-maternal interactions makes the rabbit an attractive model also for the studies of developmental origin of health and diseases (Harel et al., 1972; McArdle et al., 2009; Fisher et al., 2012).

In the present study we have assessed plasma concentrations of leptin, insulin, triiodothyronine (T3), cortisol, glucose, and non-esterified fatty acid (NEFA), as well as insulin sensitivity in pregnant rabbits to validate this species as an animal model for studying

nutritional-related disorders of pregnancy. We also hypothesized that moderate food restriction would perturb their hormonal and metabolic profiles differently according to the gestational phase in which the restriction was applied.

2. Materials and methods

2.1. Experimental design

The subjects of the present study were twenty pregnant primiparous, non-lactating does (New Zealand White) reared in the experimental rabbit farm of the Department of Agricultural and Environmental Sciences of the University of Perugia. The rabbits were housed individually in flat deck cages, the temperature ranged from +15 to +28 °C, and the light schedule was 16 L:8 D. The experimental procedures were carried out according to recommendations of the IRRG (2005). Ovulation was induced by injection of 10 μg of synthetic GnRH (Receptal, Hoechst-Roussel Vet, Milan, Italy) just before artificial insemination (AI) (Brecchia et al., 2006). The day of AI was designated as day 0. Pregnancy was diagnosed by manual palpation ten days after AI. Non-pregnant does were excluded from the experiment. The pregnant does were randomly assigned to four groups (5 does/group) according to the nutritional treatment (Table 1). The rabbits of the control group (C) were fed a standard ration (130 g/d) of commercial food (10.9 MJ ED/kg; crude protein =18.7%) throughout the gestation period (Maertens, 1993). The rabbits of the other three groups were fed a reduced amount (30% restriction, 90 g/d) of the same food, from day 0 to 9 (early, R1), from day 9 to 18 (mid, R2), and from day 19 to 28 (late, R3) of pregnancy. Before and after these restriction periods the does were fed the same standard daily ration of controls. Food intake was recorded daily until the end of pregnancy and all does consumed their rations completely.

2.2. Blood sampling

Blood samples were collected from the marginal ear vein at 0, 4, 10, 14, 18, 22, and 26 days of pregnancy, drawn into tubes containing EDTA, and immediately centrifuged at 3000xg for 15 min. Plasma was stored frozen until assayed for hormones and metabolites.

2.3. Measurements of hormones and metabolites

Plasma leptin, insulin, T3, and cortisol concentrations were determined by RIA, as reported elsewhere (Rommers et al., 2004b; Brecchia et al., 2006). Leptin concentrations were measured by double antibody RIA using the multi-species leptin kit (Linco Research Inc., St. Charles, MO, USA). The limit of sensitivity was 1.0 ng/ml and intra- and inter-assay coefficients of variations were 3.4 and 8.7%, respectively. 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 labelled 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. Total T3 was assayed by RIA according to the procedure provided by the manufacturer (Izotop, Budapest, Hungary). The sensitivity of the assay was 0.13 ng/ml, and the intra- and inter-assay coefficients of variations were 4.9 and 6.1%, respectively. Cortisol concentrations were evaluated by RIA, using the CORT kit (Immunotech, Prague, Czech Republic). The limit of sensitivity was 10 nM and intra- and inter-assay coefficients of variations were 5.8 and 9.2%, respectively.

The 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. Glucose was analyzed by the glucose oxidase method using the Glucose Infinity kit from Sigma (Sigma Diagnostic Inc., St. Louis, MO, USA).

2.4. Evaluation of insulin sensitivity

Insulin sensitivity was calculated by the homeostasis model assessment for insulin resistance (HOMA-IR) using the following equation: [insulin (mU/l) x (glucose (mg/dl)/18)]/22.5 (Helfenstein et al., 2011). Low HOMA-IR values indicate high insulin sensitivity, whereas high HOMA-IR values indicate low insulin sensitivity (insulin resistance).

2.5. Statistical analysis

One-way analysis of variance (ANOVA) followed by Bonferroni's test was performed to compare hormones and metabolite levels at different time points in the control group. The differences among groups for the variables studied were evaluated by two-way ANOVA (with group, time, and group by time effects), followed by Bonferroni's test. All statistical analyses were performed with GraphPad Prism software 5.0 (San Diego, CA, USA). Statistical significance was set at a $p < 0.05$.

3. Results

3.1. Leptin and insulin

In control does, the day of gestation affected both plasma leptin and insulin concentrations ($p < 0.001$). Compared to day 0, leptin and insulin concentrations increased ($p < 0.05$) during mid pregnancy up to day 14. Thereafter, leptin returned to basal values found at day 0, while insulin remained high ($p < 0.05$) during late pregnancy (Fig. 1, A and B). Feed restriction altered ($p < 0.001$) the pattern of leptin concentrations, whose values were lower ($p < 0.01$) in R1 and R2 than in the control group during early and mid-pregnancy (Fig. 1, A). The mean plasma concentrations of leptin during pregnancy (0 – 26 days) were lower ($p < 0.05$) in all restricted groups (1.3, 1.7, and 1.9 ng/ml in R1, R2 and R3 groups, respectively) than in control group (2.3 ng/ml). Group was not significant for insulin concentrations

although an interaction between time and group was observed ($p < 0.05$) (Fig. 1, B). Moreover, compared to control, post-hoc analysis showed a reduction of insulin concentration ($p < 0.05$) at days 10, 14, and 22 in R1, R2, and R3 groups, respectively.

3.2. T3 and cortisol

In does fed the standard diet, T3 concentrations did not change during pregnancy, while cortisol concentrations doubled ($p < 0.001$) in late pregnancy. Conversely, T3 differed between groups ($p < 0.01$), whereas cortisol concentrations were not consistently affected by food restriction (Fig. 2, B). T3 concentration was lower at day 4 in R1 ($p < 0.01$), at days 18, 22 and 26 in R2 ($p < 0.05$), and at day 22 in R3 ($p < 0.05$) group (Fig. 2, A). Moreover, mean T3 concentrations (0-26 d) were lower in R1 (162.8 ng/dl; $p < 0.01$) and R2 (145.8 ng/dl; $p < 0.001$) groups than in the control group (197.7 ng/dl).

3.3. NEFA and glucose

In control does, NEFA concentrations were affected by day of gestation ($p < 0.001$) and at day 18 the increase was significant ($p < 0.01$) compared to day 0. Conversely, glucose levels were not affected by the day of gestation. Both NEFA ($p < 0.01$) and glucose ($p < 0.001$) were affected by food restriction (Fig. 3, A and B). Compared to control does, NEFA concentrations increased by 50% ($p < 0.01$) in R3 does during the restriction period and an interaction between group and gestational day was detected ($p < 0.05$). Mean glucose concentrations in pregnancy (0-26 d) differed between control and restricted groups ($p < 0.001$): the R1 group showed higher mean values (135.6 mg/dl; $p < 0.01$) than those of the control group (117.7 mg/dl), whereas the R2 (99.3 mg/dl; $p < 0.01$) and R3 (100.4 mg/dl; $p < 0.05$) groups showed lower values than those of the control group.

3.4. Insulin sensitivity

HOMA-IR was affected by day ($p < 0.001$), group ($p < 0.001$), and their interaction ($p < 0.05$). In control does, HOMA-IR indexes showed an increase at days 14 ($p < 0.05$) and 22 ($p < 0.01$) of pregnancy, when compared with day 0. Feed restriction reduced the values in R2 ($p < 0.05$) and R3 ($p < 0.01$) of the HOMA-IR indexes during restriction periods (Table 2).

4. Discussion and conclusions

In rabbits, pregnancy induced a well coordinated range of hormonal and metabolic adaptations necessary to fulfil the energy requirements of both mother and foetuses. Moderate and transitory food restriction during pregnancy altered the energy homeostasis with adaptive changes to the hormonal and metabolic environment of pregnancy.

Leptin is primarily an adipose-derived hormone that regulates energy homeostasis by modulating food intake and energy expenditure (Woods et al., 1998; Ladyman, 2008; Brecchia et al., 2010). During pregnancy, leptin plays multiple roles including the development in the foetus of the neuronal hypothalamic network involved in energy homeostasis (Hauguel-de Mouzon et al., 2006; Desai et al., 2011). In rabbits, the profile of leptin concentration during pregnancy was similar to that found in other species (Henson and Castracane, 2000; Block et al., 2001; Ladyman, 2008). At mid pregnancy, leptin concentrations were 68% higher than those found at insemination. This increase is similar to that found in humans (+30%), but much lower than that of rats (2-fold rise) and mice (20- to 40-fold rise) (Henson and Castracane, 2000; Hauguel-de Mouzon et al., 2006). The mechanism by which peripheral plasma leptin concentrations increase as well as its likely site of synthesis and release during pregnancy are still unclear, but, both in humans and rodents, hyperleptinemia is associated with hyperphagia.

Interestingly, toward the end of gestation leptin concentrations decreased, as also reported in ruminants (Block et al., 2001) and humans (Henson and Castracane, 2000). This leptin decrease should have stimulated food consumption, but instead is associated with anorexia

that occurs two-three days preceding the birth (Fortun-Lamothe, 2006; Manal et al., 2010). The decreasing plasma concentrations of leptin at the end of gestation may reflect the negative energy balance at this stage of pregnancy and may coordinate the neuroendocrine adaptations responsible for partitioning energy from mother to growing foetuses and/or anticipate the increase of food consumption during lactation (Block et al., 2001). In the present study, feed restriction during pregnancy hampered the increase of leptin found in normally fed does, consistent with signalling an energy deficit. Similar results have been reported in rodents (Delahaye et al., 2008).

The best known action of insulin is the control of intermediary metabolism, especially glucose homeostasis. However, insulin has several properties as an adiposity signal (Woods et al., 1998; Brecchia et al., 2010) and may be also implicated in metabolic programming of offspring (Ozanne et al., 2005; Warner and Ozanne, 2010; Tamashiro and Moran, 2010; Desai et al., 2011). During pregnancy, insulin concentrations increased in does fed the standard diet, a finding in agreement with previous studies (Hauguel et al., 1987; Fortun-Lamothe, 2006). In human beings, it is well established that normal pregnancy is associated with insulin resistance (Ciampelli et al., 1998; Sivan et al., 1999). The current study did not include a direct measurement of insulin resistance, such as the oral glucose tolerance test, but the increase in NEFA, reflecting the decreased ability of insulin to suppress lipolysis (Sivan et al., 1999), as well as that of the HOMA-IR, confirm low insulin sensitivity in mid and late rabbit gestation (Hauguel et al., 1987). Taken together, the increase of blood insulin concentrations and insulin resistance may represent an adaptive mechanism to cope with the increasing demands of the foetuses. In fact, the reduction of insulin-dependent glucose utilization by tissues such as muscles could contribute to the repartition of nutrients between the mother and foetuses via the placenta (Hauguel et al., 1987; Ciampelli et al., 1998; Sivan et al., 1999; Diderholm et al., 2006). In our study insulin concentrations decreased, although not very significantly, during food restriction suggesting a physiological adjustment for restoring

energy homeostasis. In fact, the reduced levels of insulin sub-serve glycogenolysis, *de novo* hepatic synthesis of glucose, and, like leptin, contribute to central neural control of food intake (Woods et al., 1998; Brecchia et al., 2010).

T3 is a key hormone in the regulation of metabolism and in adaptation to fasting: it contributes to both obligatory and adaptive thermogenesis, regulating appetite and energy expenditure. Our study showed substantially unchanged levels of total T3 during normal pregnancy, but conditions of reduced nutrition had a significant influence. The T3 decrease during the restriction periods reduces the basal metabolic rate, resulting in energy savings for the animals. In non pregnant rabbits, T3 plasma concentrations were markedly reduced during short-term fasting (Brecchia et al., 2006), while in newborn rabbits, with limited thermogenic capabilities, the thyroid axis was only marginally affected by the reduction in energy intake (Brecchia et al., 2010).

Glucocorticoids are essential for the development and maturation of foetal organs (Tamashiro and Moran, 2010) as well as for the events related to parturition (Mastorakos and Ilias, 2003). The 2-fold cortisol increase in late pregnancy confirmed the data reported in several other species including human beings (Mastorakos and Ilias, 2003; Brunton et al., 2008). Unexpectedly, though, cortisol was not affected by food restriction. Cortisol secretion is a generic response to stress as well as a specific adaptive response to fasting and nutritional stress recruiting all available energy sources in the body (Mastorakos and Ilias, 2003; Brunton et al., 2008; Brecchia et al., 2009). However, several studies have shown that stress-induced activation of the hypothalamic-pituitary-adrenal axis is altered during pregnancy (Brunton et al., 2008; Slattery and Neumann, 2008; Entringer et al., 2010). In women, administration of exogenous corticotrophin-releasing hormone in late pregnancy failed to evoke a significant adrenal response (Schulte et al., 1990). It has been suggested that this hyporesponsiveness to stress is one of the adaptive mechanisms occurring during pregnancy to protect the foetus from excess glucocorticoid exposure (Brunton et al., 2008; Slattery and Neumann, 2008) that

would later cause deleterious effects on adult cardiovascular, metabolic and neurobehavioural phenotypes (Mastorakos and Ilias, 2003; Brecchia et al., 2009; Tamashiro and Moran, 2010).

In rabbits fed the standard diet, the increase of plasma NEFA concentration indicates the mobilization of energy reserves during late pregnancy (Fortun-Lamothe, 2006), in agreement with leptin reduction and insulin resistance. In pregnant women, there is an increase of almost 50 per cent in the rate of lipolysis (Diderholm et al., 2005). The energy from lipolysis favours gluconeogenesis, thus saving glucose and amino acids for the growing foetus (Sivan et al., 1999; Diderholm et al., 2006). Therefore, moderate NEFA increase in late pregnancy can be considered an adaptation for nutrient partitioning. However, high NEFA levels are also a marker of severe negative energy balance and pregnancy disorders (Adewuyi et al., 2005; Villa et al., 2009; Ortega-Senovilla et al., 2010; Martínez-Paredes et al. 2012). In human beings, there is a relationship between high maternal NEFA concentrations and preeclampsia, reduced intrauterine growth, and low birth weight. In addition, high NEFA levels are factors predisposing to an increased risk of adult diseases (Villa et al., 2009; Ortega-Senovilla et al., 2010).

In the present study, food restriction during early and mid gestation did not change NEFA concentrations despite the low insulin concentrations. In non pregnant rabbits, several authors demonstrated an increase of lipolysis and NEFA concentrations during fasting (Brecchia et al., 2006; Weber and Reidy, 2012). In our experiment, however, there was no complete food deprivation and moreover, the energy balance of the first week of rabbit pregnancy is positive because the requirements for foetal growth are relatively small (Fortun-Lamothe, 2006). Conversely, the NEFA increase was marked (+50%) in does subjected to food restriction in late pregnancy. High NEFA levels, associated with low circulating insulin, indicate the critical mobilization of body reserves when food restriction occurs in the most energetically expensive phase of gestation.

In this study, glucose was not influenced by day of gestation in the control group. This result indicates that, in well-fed rabbits, the fine homeostatic endocrine mechanisms described above maintain constant concentrations of this critical metabolite during pregnancy. Instead, food restriction affected mean glucose plasma concentrations differently, depending on the gestational phase. Restriction during early pregnancy increased mean glucose concentrations, dietary restriction during mid and late pregnancy had an opposite effect. We presume that hormonal responses to food restriction are efficient during a phase of positive energy balance (early pregnancy) in mobilizing glycogen reserves so that glucose homeostasis is preserved. During mid and late pregnancy, the foetuses use a considerable amount of maternal glucose (Diderholm et al., 2006; Fortun-Lamothe, 2006). If nutritional requirements are not met in these phases, glucose homeostatic regulation fails and the mean glucose level decreases. Low glucose concentrations and increased NEFA may be associated with the greater energy deficit in rabbits subjected to food restriction in late pregnancy. The difference in energy requirements in each phase of gestation is relevant because, in addition to hormonal internal environment, reduced supply of energy substrates to foetuses could be one factor underlying metabolic programming (Diderholm et al., 2006; Warner and Ozanne, 2010).

In conclusion, we have found that hormonal and metabolic profiles of rabbit pregnancy reveal several analogies with those of women. In addition, we have demonstrated that food restriction alters some hormonal and metabolic parameters involved in predisposition to adult diseases in different ways, depending on gestational phase in which it is applied. Our study lays the groundwork for further studies on metabolic programming using the rabbit as an experimental model.

Acknowledgements. The authors gratefully acknowledge the revision of the English text by Sheila Beatty. This work is part of the PhD program in "Animal health, Livestock production and food safety" XXVIII cycle.

5. References

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Table 1. Experimental schedule. Feeding plans of control group (C; n=5) and of does subjected to restriction in early (R1; n=5), mid (R2; n=5), and late (R3; n=5) pregnancy. The gray rectangles indicate feeding with standard ration (130 g/d) while black rectangles indicate periods of food restriction (90 g/d) for each group. Before AI and after kindling, all the animals ate the standard ration.

Group	n° of animals	Day of pregnancy		
		0-9	9-18	19-28
C	5			
R1	5			
R2	5			
R3	5			

Legend

130 g/d 90 g/d

Table 2. HOMA-IR levels in control (C, n = 5) and restricted groups (R1, n = 5; R2, n = 5; R3, n = 5) during pregnancy. Values are means ± S.E.M.. The shaded areas indicate the feed restriction periods of each group (90 g/d for 10 days): from day 0 to 9 (R1), from day 9 to 18 (R2), and from day 19 to 28 (R3). Before and after these restriction periods the does were fed the standard ration (130 g/d).

Group	Day of pregnancy						
	0	**4**	**10**	**14**	**18**	**22**	**26**
C	7.3±0.4	9.8±0.6	13.2±0.3	16.6±1.7$^{\#}$	10.5±1.3	20.1±3.5$^{\#\#}$	14.2±1.4
R1	10.0±1.1	9.9±1.6	8.0±0.8	14.6±1.4	17.6±1.4	22.9±2.9	13.9±0.4
R2	6.5±0.5	7.3±1.6	10.9±2.8	8.9±1.3*	8.5±3.2	16.6±1.1	12.1±0.5
R3	7.2±1.5	6.9±4.6	12.0±1.3	13.5±1.4	9.9±2.4	9.6±0.2**	8.9±2.1

$^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ control day *versus* day 0;

* $p < 0.05$, ** $p < 0.01$ restricted group *versus* control group for each gestation day.

Captions for figures

Figure 1. Leptin (Panel A) and insulin (Panel B) concentrations during pregnancy in control (C, n=5) and restricted groups (R1, n=5; R2, n=5; R3, n=5). Values are means ± S.E.M.. For clarity, only positive error bars are shown. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.01$ control day *versus* day 0; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ restricted group *versus* control group for each gestation day (Bonferroni's post-test).

Figure 2. T3 (Panel A) and cortisol (Panel B) concentrations during pregnancy in control (C, n=5) and restricted groups (R1, n=5; R2, n=5; R3, n=5). Values are means ± S.E.M.. For clarity, only positive error bars are shown. ### $p < 0.001$ control day *versus* day 0; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ restricted group *versus* control group for each gestation day (Bonferroni's post-test).

Figure 3. NEFA (Panel A) and glucose (Panel B) concentrations during pregnancy in control (C, n=5) and restricted groups (R1, n=5; R2, n=5; R3, n=5). Values are means ± S.E.M.. For clarity, only positive error bars are shown. ## $p < 0.01$ control day *versus* day 0; ** $p < 0.01$ restricted group *versus* control group for each gestation day (Bonferroni's post-test).

Figure 1 A

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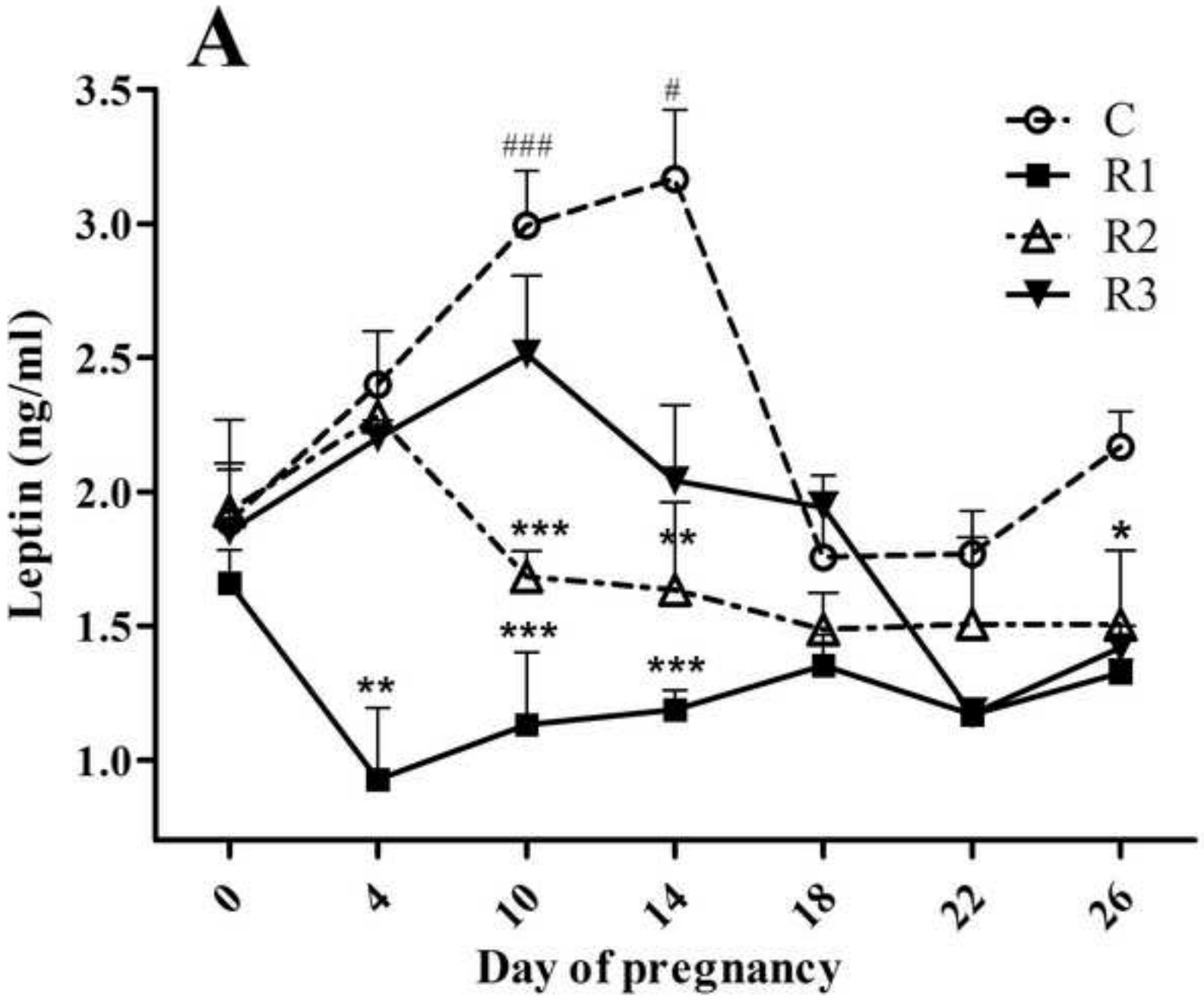

Figure 1 B

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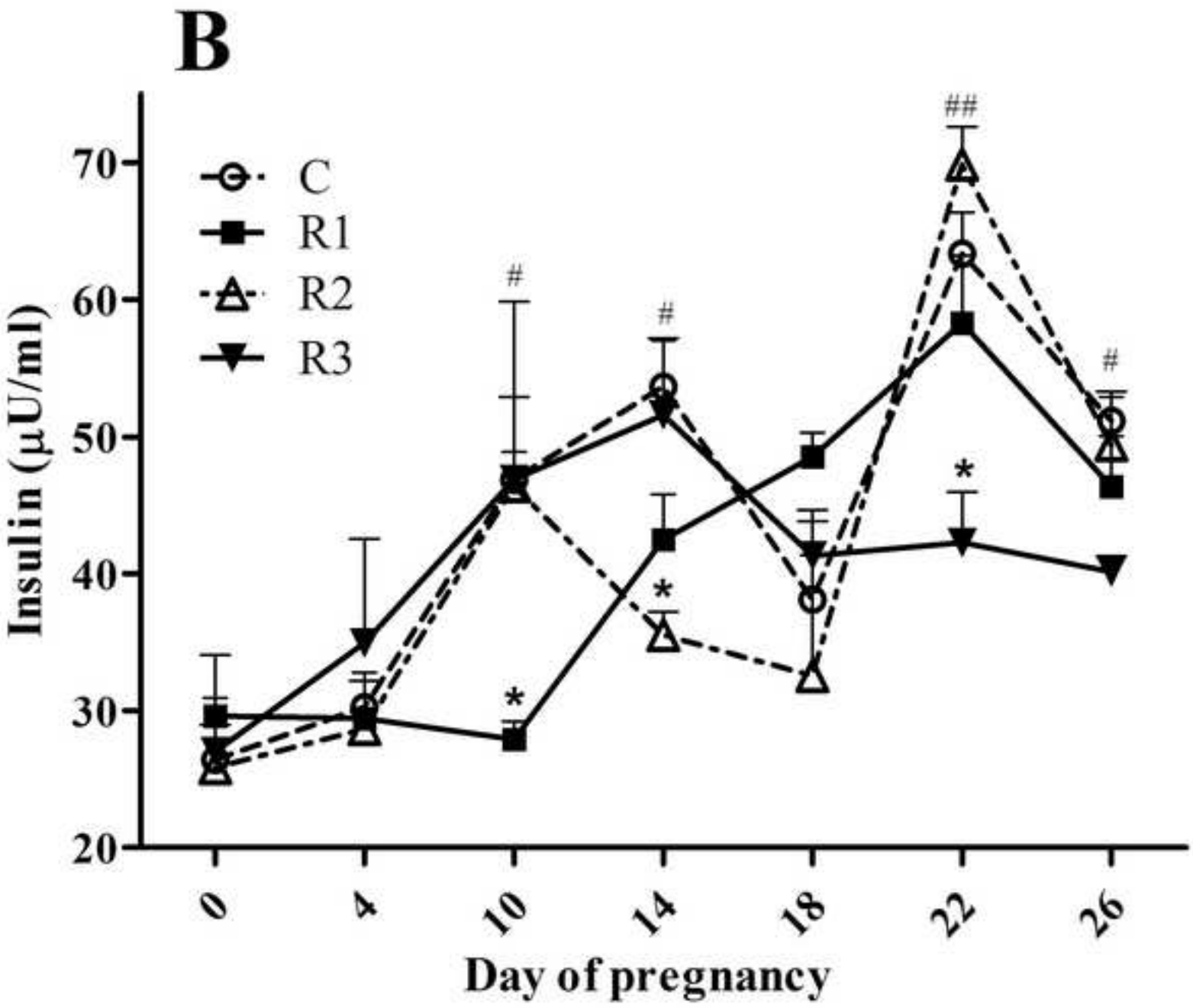

Figure 2 A

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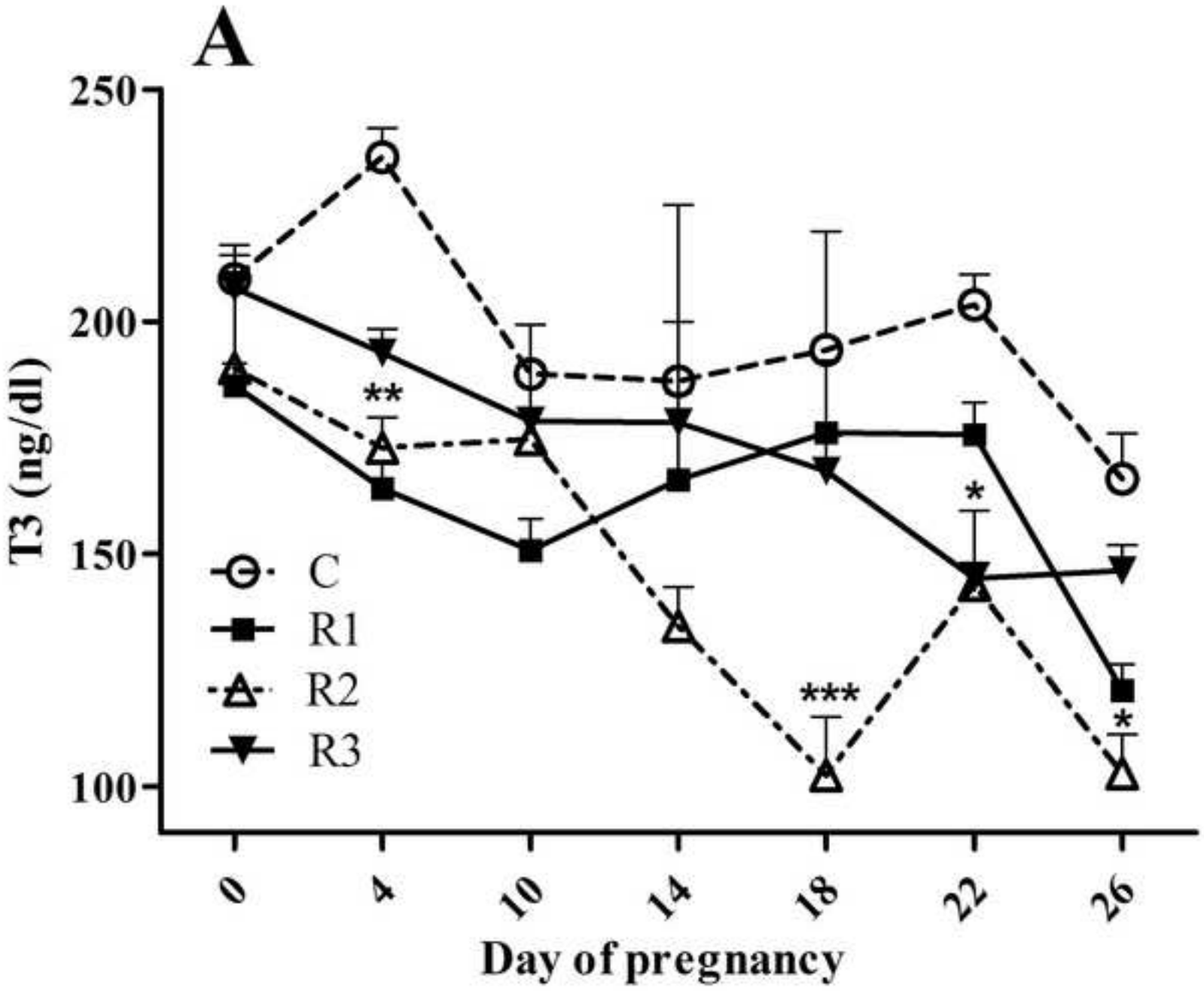

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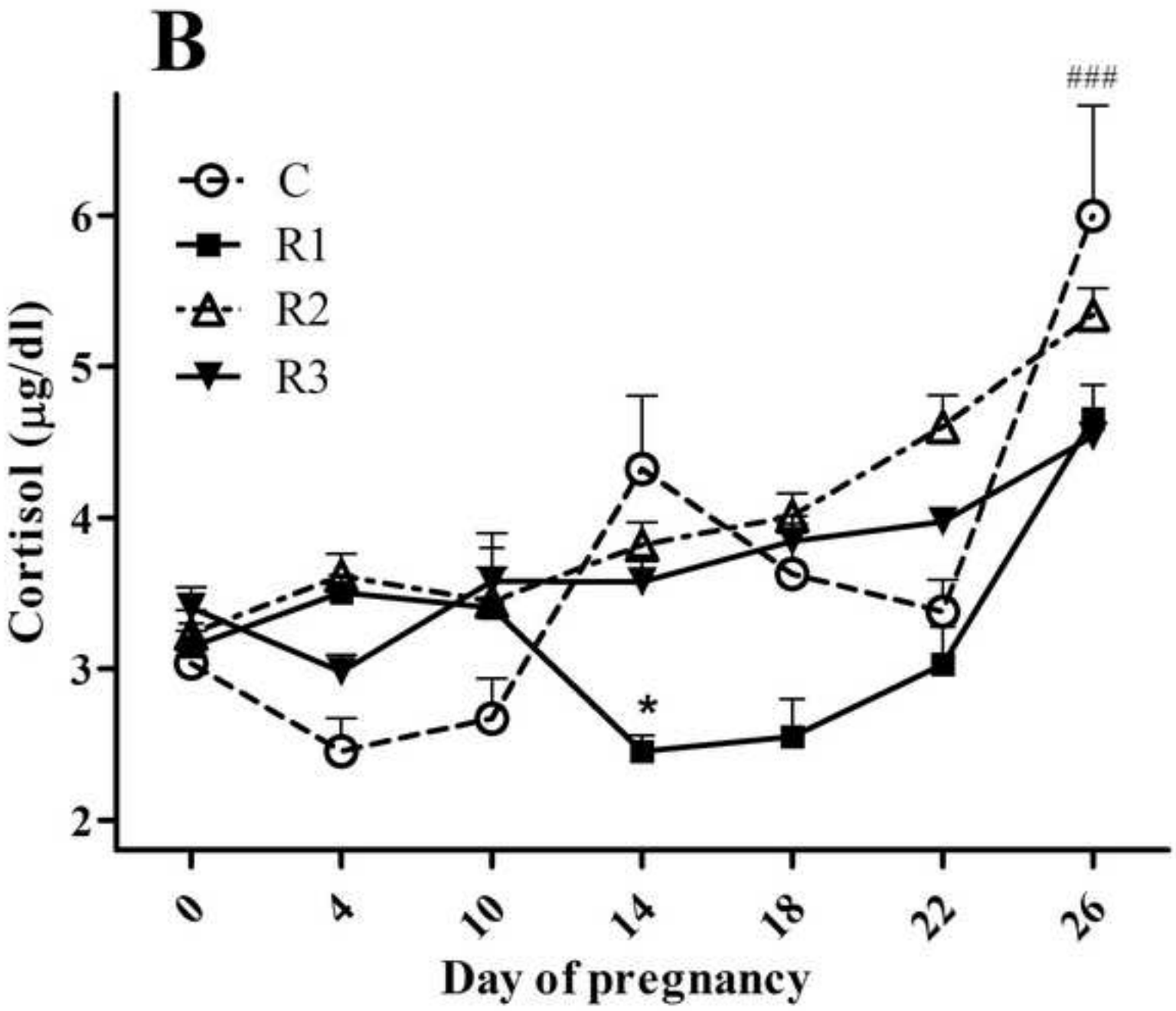

Figure 3 A

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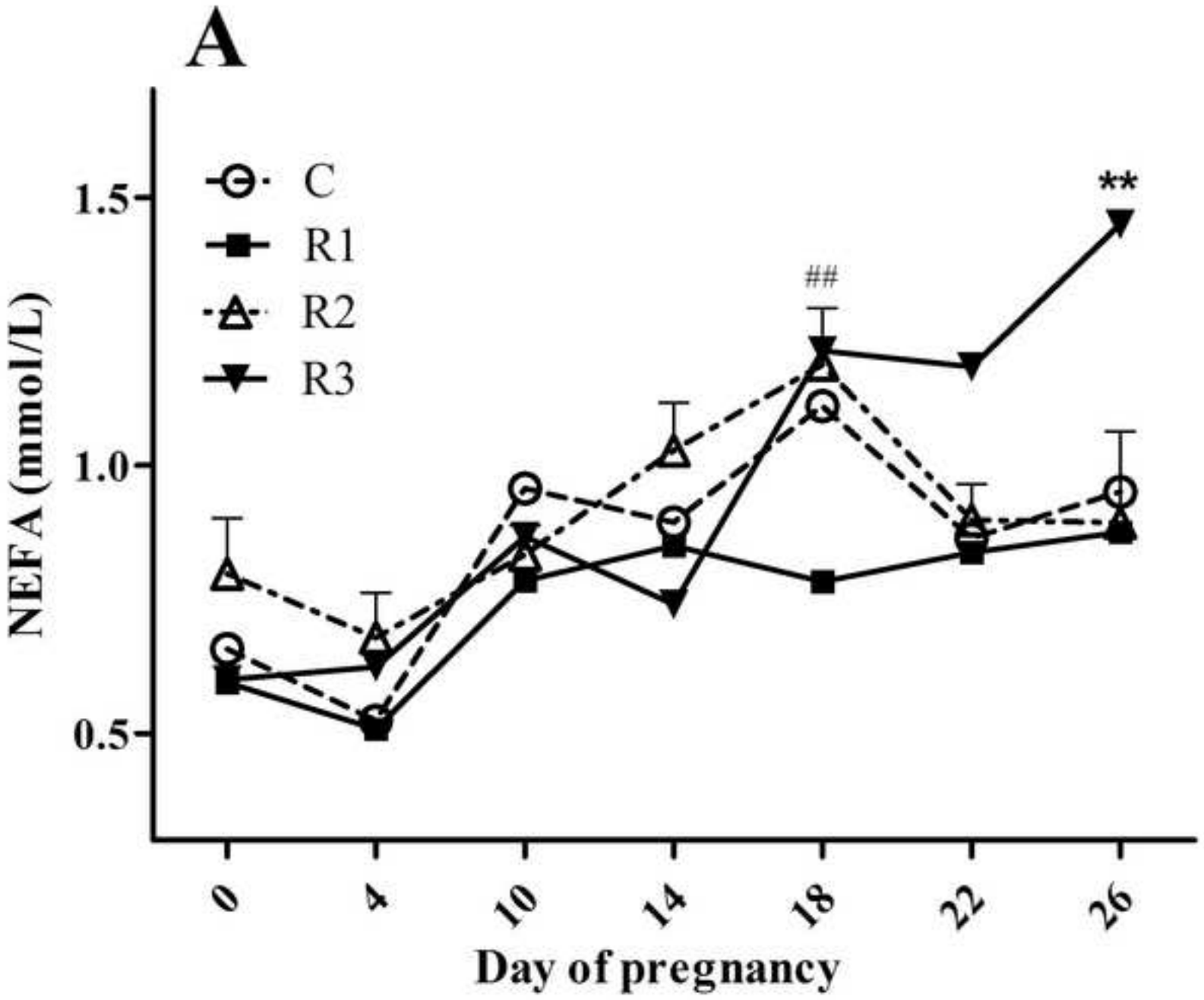

Figure 3 B

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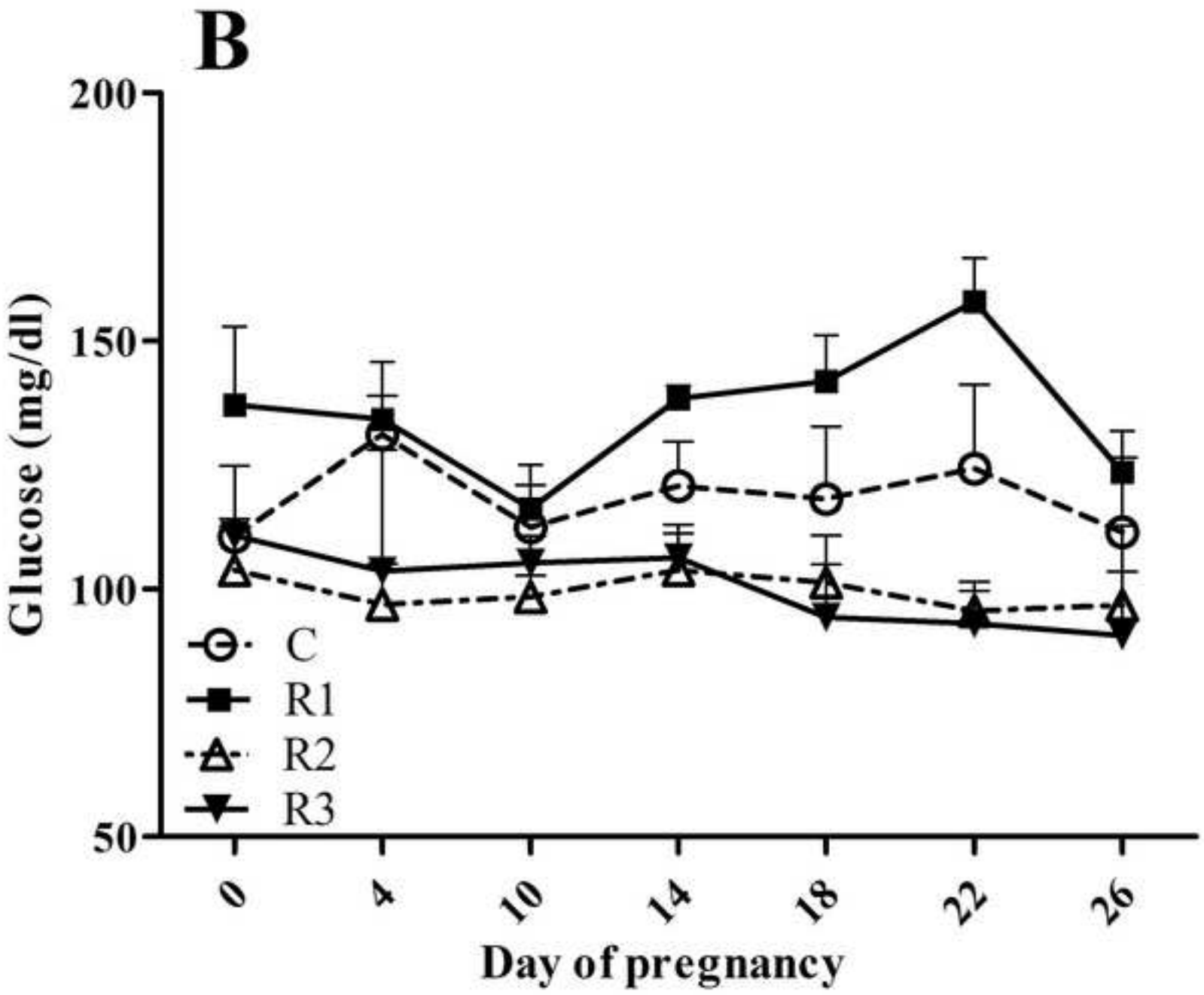

***Highlights**

- Pregnancy induces hormonal adaptations to fulfil energy requirements
- Food restriction alters hormones and metabolites involved in metabolic programming
- Changes depend on the gestational phase of food restriction
- The rabbit is an attractive model to study nutritional-related disorders of pregnancy