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Non-invasive endocrine monitoring of ovarian and adrenal activity in chinchilla (*Chinchilla lanigera*) females during pregnancy, parturition and early post-partum period

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ABSTRACT

The chinchilla is a rodent that bears one of the finest and most valuable pelts in the world. The wild counterpart is, however, almost extinct because of a drastic past and ongoing population decline. The present work was developed to increase our knowledge of the reproductive physiology of pregnancy and post-partum estrus in the chinchilla, characterizing the endocrine patterns of urinary progesterone, estradiol, LH and cortisol metabolites throughout gestation and post-partum estrus and estimating the ovulation timing at post-partum estrus.

Longitudinal urine samples were collected once per week throughout pregnancy and analyzed for creatinine, cortisol, LH, estrogen and progesterone metabolite concentrations. To indirectly determine the ovulation timing at post-partum estrus, a second experiment was performed using pregnant females subjected to a post-partum *in vivo* fertilization scheme. Urinary progestagen metabolites increased above baseline levels in early pregnancy between weeks-8 and -11 respectively to parturition, and slightly declined at parturition time. Urinary estrogens showed rising levels throughout mid- and late pregnancy (weeks-9 to -6 and a further increase at week-5 to parturition) and decreased in a stepwise manner after parturition, returning to baseline levels two weeks thereafter. Cortisol metabolite levels were relatively constant throughout pregnancy with a tendency for higher levels in the last third of gestation and after the pups' birth. Parturition was associated with dramatic reductions in urinary concentrations of sex steroids (especially progestagens). Observations in breeding farms indicated that the females that resulted in a second pregnancy after mating, did so on the second day after parturition. These data were in agreement with an LH peak detected 24 h after parturition. Urinary steroid hormone patterns of estrogen and progestagen metabolites provided valuable information on endocrine events during pregnancy and after parturition in the chinchilla. Results presented in this study enhance our understanding of natural reproductive dynamics in the chinchilla and support empirical observations of breeders that post-partum ovulation occurs ~48 h after parturition.

1. Introduction

The chinchilla is a South American rodent that bears one of the finest and most valuable pelts in the world; a hybrid of the two chinchilla species (*Chinchilla lanigera* and *Chinchilla brevicaudata*) has been domesticated, bred and selected for fur quality, color and growth rate for over 80 years (Grau, 1986). The wild counterpart was, however, almost extirpated during the early 1900s due to severe poaching that led to a drastic population decline, estimated to be more than 90% of their original numbers. Remnant populations remain in reserves of central Chile, yet the number and size of colonies are decreasing and becoming more fragmented (Jiménez, 1996). Currently, *Chinchilla* species are included in Appendix I of CITES and considered Critically Endangered by the IUCN (IUCN Red Book, 2008; CITES, 1973), although little international attention has been paid to the serious status of the species.

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In light of the situation for this species, the need for immediate conservation actions cannot be overstated: without active management, research and conservation, wild chinchilla populations are likely to become extinct in the near future (Jiménez, 1994). It is clear that successful conservation programs must utilize active management techniques to enhance genetic diversity of the extant population. This could be facilitated by incorporating captive breeding programs and assisted reproductive techniques into a global management plan. However, implementing effective assisted breeding programs depends on a thorough knowledge of the species' reproductive physiology that is, at present, either unknown or poorly studied in the chinchilla (Ponzio et al., 2007).

In this aspect, the domestic chinchilla offers a unique opportunity: it can be a useful model to obtain knowledge that will be directly applicable to their wild counterparts (Ponzio et al., 2007; Busso et al., 2012).

Several aspects of chinchilla male reproductive physiology have been studied by our research team (Ponce et al., 1998; Carrascosa et al., 2001; Busso et al., 2005a, 2005b, 2005c; Ponzio et al., 2007, 2008, 2011). A logical extension is to expand our efforts towards the characterization of female reproductive physiology of which very little is known: the estrous cycle has been found to range from 15 to 90 days using various technical approaches (Busso et al., 2012); females exhibit spontaneous ovulation, typically with increased estrus observed in winter that is consistent with a high birth rate in spring (Weir, 1970; Grau, 1986). Vaginal opening, an indication of estrus, occurs during a period of 7–8 days (Weir, 1970), but no precise data are available regarding the timing of ovulation.

Females typically twin (range: 1–6 pups) after a gestation of ~112 days (range: 105–118 days) and give birth to 2–3 litters per year, resulting in relatively few offspring compared to other rodent species. A post-partum estrus occurs during the first 24–72 h following parturition, and if the female conceives, post-partum mating will simultaneously result in gestation and lactation of two different litters (Neira et al., 1989; Spotorno et al., 2004). However, our knowledge of the regulation of endocrine events and hormonal profiles occurring during gestation and post-partum estrus in the chinchilla remains poorly defined.

Therefore, the overall goal of the present work was to increase our knowledge of the reproductive physiology of pregnancy and post-partum estrus in the chinchilla. The specific objectives were to: (1) evaluate the endocrine patterns of urinary progesterone, estradiol, LH and cortisol metabolites throughout gestation and post-partum estrus; (2) indirectly estimate the ovulation timing at post-partum estrus.

2. Materials and methods

2.1. Animals and housing

Fifteen sexually mature domestic chinchilla (*C. lanigera*) females and 3 males were used in this study (age range: 2–5 years). Animals were housed at our chinchilla breeding facility one month before the onset of the experiments in a classic polygamous breeding system (1 male per 5 females). Each female was allocated in individual stainless steel cages (32 cm wide × 30 cm high × 50 cm length) with wood shaving substrate and a corridor in the back that allowed the male to enter any of the female cages in the family. This corridor remains closed to the male until the beginning of the experiment, allowing close olfactory and visual contact but not copulation.

The animals were exposed to natural fluctuations in photoperiod (Córdoba, Argentina, 31° S–64° W; winter and summer solstice 10.1 and 14.2 h light/day, respectively) and controlled temperature (20–23 °C). Pelleted chinchilla food (Chinworld, Escobar, Buenos Aires, Argentina) and water were provided *ad libitum*, and a cube of compressed alfalfa was fed once weekly. A tablespoon of marble powder was added to the substrate of each cage on a regular basis for the animals to perform a "dust bath" for maintaining dry and uncompressed fur.

To determine the *in vivo* conception rates on specific days during post-partum estrus (as an estimation of the timing of post-partum ovulation), a second experiment was performed using 86 females maintained at five commercial farms. The housing, feeding and routine management conditions provided to the animals in the farms were the same as in the laboratory.

In all cases, adequate measures were taken to minimize animal discomfort or pain, and experiments were conducted in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals.

2.2. Experimental design

2.2.1. Longitudinal endocrine monitoring

After the habituation period, the corridors were opened and animals were allowed to mate. Longitudinal urine samples were then collected from each female once per week throughout pregnancy up to approximately 3–4 weeks before the expected time of parturition (as determined by weekly abdominal palpation of the pups' growth and movement at the time of allocation of the female to the metabolic cage). Thereafter and for 15 days post-partum, females were allocated permanently to the metabolic cages and urine samples were collected daily. All samples were stored at −20 °C until analyzed for creatinine, cortisol, luteinizing hormone (LH), estrogen and progesterone metabolite concentrations.

The date of conception was estimated retrospectively at full-term delivery. Animals that failed to conceive as determined by the absence of increased body weight or the birth of live offspring were not included in the study. Increase in body weight was also recorded once per week.

2.2.2. In vivo conception rates at post-partum estrus

In a second experiment, two methods were employed to indirectly determine the ovulation timing at post-partum estrus using animals located in commercial breeding farms. Pregnant females at weeks 12–15 of gestation (determined by body weight increase and abdominal palpation of the pups) were selected and randomly assigned to five experimental groups. All females were observed each day at 7–9 a.m. until the birth of live offspring occurred (births in chinchillas usually occur during early morning hours). Animals were then allowed to mate for 24 consecutive hours (after parturition) according to the following scheme: Group 1: copulation was allowed immediately after parturition; Group 2: copulation was allowed only after 48 h; Group 3: copulation was allowed only after 72 h; Group 4: copulation was allowed only after 96 h; Group 5: copulation was allowed only after 120 h; Control: copulation was allowed for 10 days. Percentages of females fertilized at different times of post-partum estrus in each group were therefore determined after a second gestation period, and birth of a post-partum litter.

Additionally, reproductive databases of 217 females maintained in local farms were analyzed, considering those females that presented a second litter after the occurrence of the post-partum estrus (corroborated by the birth of a second litter ~112 days after). To estimate the fertilization time, we considered the date of birth of a first and second consecutive litter in each female. The mean gestation length in this group of females (mean number of days between first and second litter) was then subtracted from the time of birth of the second litter, to estimate when the fertilization of the female occurred.

2.3. Urine collection

To separate urine and feces at time of excretion, metabolic cages were constructed by making slight modifications to a regular housing cage. Cage bottoms were triple-layered steel litter pans. The top pan had transversal steel rods that provided a supportive surface for the animal. The middle pan consisted of a steel mesh (1.0 mm diameter openings) that permitted urine, but not feces, to pass through to the lower pan, which formed a steel funnel that directed the urine into a collection tube containing 500 μl of 70% ethanol as preservative. To avoid discomfort of the animals because of the grid floor in the metabolic cages, a platform was added in the upper back of the cage to provide a solid resting area (32 × 14 cm). This element was provided to the animals in the regular housing cages also, both in the laboratory and in commercial breeding conditions. Each urine sample was collected overnight (chinchillas usually urinate only during nighttime hours; 18:00–09:00 h), centrifuged to separate detritus and stored at −20 °C until processing.

2.4. Urinary steroid analysis

Immunoreactive concentrations of urinary metabolites of estradiol (E_2) and progesterone (P_4) were measured in all samples collected throughout gestation and post-partum estrus, using commercial ^{125}I-RIA kits for the determination of those hormones in human serum or plasma (Coat-A-Count, Siemens, Buenos Aires, Argentina). Profiles of urinary estrone conjugates (EC), pregnandiol-3-glucuronides (PdG) and cortisol were determined by EIA using polyclonal antibodies, standards and their corresponding horseradish peroxidase conjugates (R522–2, R13904 and R4866 respectively, Department of Population Health and Reproduction, C. Munro, UC Davis, CA, USA). Urinary LH metabolites were also determined in all samples using an in-house I^{125} RIA for LH determination in rat serum (NIDDK-anti rat LH-S118 AFPC697071P, raised in rabbits, National Hormone & Peptide Program, USA).

The E_2, P_4 and cortisol assays have been previously validated and described in full by our group (Busso et al., 2007; Ponzio et al., 2004). Validations for EC and PdG EIAs, and LH RIA in chinchilla urine were conducted and reported here by demonstrating: (1) parallelism between dilutions of pooled urine and the respective standard curves, in order to detect immunological similarities between the standard and sample hormones (EC: $r = 0.97$; PdG: $r = 0.99$; LH: r = 0.84; $p < 0.01$) and (2) significant recovery (>90%) of exogenous hormone in the range of the standard curve (EC: 0.039 to 5.0 ng/ml; PdG: 1.96 to 250 ng/ml) added to the urine sample, in order to examine possible interference of components in urine with antibody binding (EC: 97.1 ± 9.8%, $y = 1.15x - 4.86$, $r^2 = 0.997$; PdG: 91.3 ± 2.2%, $y = 0.97x - 0.18$, $r^2 = 0.996$; LH: $y = 2.62x + 0.79$, $r^2 = 0.96$; $p < 0.01$).

Prior to EC and PdG assay and according to the parallelism results, urine samples were diluted at 1:32 with EIA buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of BSA per liter) and assayed in duplicate. Flat-bottom microtiter plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada) were first coated with 50 μl of the antibody stock diluted in coating buffer (50 mM bicarbonate buffer, pH 9.6; 1:13300 for EC and 1:10240 for PdG), covered with acetate sealers to prevent evaporation and incubated overnight at 4 °C. After 16–24 h, plates were washed to remove any unbound antibody with 0.02% Tween 20 solution using a Bio-Tek ELx 405VR microplate washer (Bio-Tek Instruments, Winooski, VT). Immediately after washing, 50 μl of urine samples, standards, and controls diluted in EIA buffer were added in duplicates, followed by 50 μl of horseradish peroxidase conjugate diluted in EIA buffer (EC 1:37600; PdG 1:21530). Plates were then covered and incubated at room temperature (21 °C) for 2 h.

Following incubation, the plates were washed and blotted dry, and 100 μl of substrate solution (50 mM citrate, 1.6 mM hydrogen peroxide, and 0.4 mM 2,20-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, pH 4.0) were added to each well (Munro et al., 1991). Absorbance was measured at 405 nm using a microplate reader (MRX microplate reader, Dynex Technologies, Chantilly, VA).

For the LH assay and according to the parallelism results, urine samples were diluted at 1:2 with PBS-1% egg albumin and assayed in duplicate. Briefly (Di Giorgio et al., 2013), the first antiserum (NIDDK anti rat LH-S118 AFPC697071P) was diluted at 1:274400 in PBS, EDTA 0.05 M with 0.25% rabbit serum, pH 7.4. The reference standards were purified LH obtained from rat hypophysis (NIDDK-Rat-LH-RP3: 0.015–8 ng/tube) in PBS-1% egg albumin. The secondary antibody was ovine anti-rabbit globuline diluted at 1:500 in PBS-8% propileneglicol 6000. One hundred microliters of the first antibody and 100 μl of the tracer (NIDDK rLH-I-10, iodinated with 125-I (NEZ033H Perkin Elmer, USA): 17.000–20000 cpm/tube) were added to the standard tubes and the urine samples (100 μl each). After 24 h of incubation at room temperature, 500 μl of the secondary antibody were added to each tube. The samples were further incubated for 2 h at room temperature and for 1 h at 4 °C. After all the incubation periods, the tubes were centrifuged at 2000 rpm for 30 min at 4 °C (Beckmman Instruments). The supernatant was aspirated and the radioactivity associated to the hormone-antibody complex in the precipitate was read in a gamma counter (Packard instruments, Palo Alto, CA, USA).

Cross-reactivities for EC are: estrone 3-glucuronide 100%, estrone 3-sulfate 66.6%, estrone 238%, estradiol 17β 7.8%, estradiol 3-glucuronide 3.8%, estradiol 3-sulfate 3.3%, estradiol 17-sulfate 0.1%, estradiol 3-disulfate 0.1%, and < 0.1 with all other steroids tested.

Cross-reactivities for PdG are: pregnanediol 3-glucuronide 100%, 20α-Hydroxy-progesterone 44.8%, 20β-Hydroxy-progesterone 3.1%, progesterone 0.7%, estradiol 17β 0.04%, testosterone 0.2% and cortisol 0.06%. The cortisol antiserum crossreacts with prednisolone 9.9%, prednisone 6.3%, cortisone 5.0%, corticosterone 0.7%, 21-deoxycortisone 0.5%, deoxycorticosterone 0.3%, progesterone 0.2%, 11-desoxycortisol 0.2%, 17α-hydroxyprogesterone 0.2%, and <0.1% with all other steroids tested.

Cross-reactivities for LH are: LH 100%, thyrotropin stimulating hormone 0.13% and <0.1 % with follicle stimulating hormone, growth hormone and prolactin.

For all hormonal determinations, the accepted intra- and interassay coefficients of variation for the high and low control samples were <10 and <15%, respectively. Assay sensitivity was 0.039 ng/ml for EC, 1.96 ng/ml for PdG, 0.078 ng/ml for cortisol and 0.015 ng/ml for LH.

Due to possible variations in fluid intake and output, concentration of all hormones in urine samples was adjusted for creatinine and expressed as hormone mass per mg creatinine (colorimetric assay from creatinine standard set, #C3613, Sigma Chemical Co., St. Louis, MO, USA) (Monfort et al., 1991).

2.5. Data analysis

All values were aligned to day of parturition and expressed as mean ± standard error of mean (SEM), recognizing the fact that hormonal metabolites appear in urine a few hours later than their presence in circulating blood (Busso et al., 2007). Additionally, each hormonal data point in the graph representing weeks-3 to 3 is the mean of hormonal values from the daily samples obtained for

those particular weeks, with the exception of LH, which is presented day by day.

Data analysis was performed using the Infostat statistical software (Di Rienzo et al., 2012). For the analysis of all hormonal data and body weight, a linear mixed effects model was applied. The numerical routines implemented in InfoStat for the estimation of mixed-effects-linear models are based on the nlme package (Pinheiro et al., 2012) of R (R Core Team, 2012). The factor *gestation week* was included as a fixed effect; meanwhile the *animal* effect was included as random. The animal effect accounts for the intra-class correlation, which is a common correlation structure for repeated measures on the same experimental unit (animal). Additionally, a first-order autoregressive correlation function was added to model the correlation among time-indexed measurements. In any case the need for these correlation structures was verified by the Akaike Information Criterion.

To check the assumptions of normality and homoscedasticity, normal qq-plots and scatter plots between residuals and predicted value were controlled. Guided by these diagnostic analyses, P_4, PdG, cortisol and LH data were transformed to log scale. For the analysis of E_2, EC and body weight data, a variance function (varPower – see nlme documentation for details) was also included in the model. The DGC post hoc test (Di Rienzo et al., 2002) was applied to perform the pair-wise comparisons of adjusted means when the overall (F) test was significant. The significance level for all statistical tests was set at 0.05.

For all hormonal data, baseline levels are considered as the hormonal concentration levels at the beginning of the observations and up until a significant change in the values is detected using the linear mixed effects model.

The cortisol metabolite data were additionally analyzed considering mean hormone metabolite concentrations at early, mean and late pregnancy (~5 weeks each period), using ANOVA and the DGC post hoc test.

The correlation coefficients for estrogen and progestagen metabolite concentrations determined by RIA (E_2 and P_4) vs. EIA (EC and PdG) were determined using the Spearman correlation test.

Results from *in vivo* conception rates at post-partum estrus were analyzed using a Chi-square test.

3. Results

3.1. Longitudinal endocrine monitoring

Of the 15 mated females, 8 conceived and successfully gave birth to live offspring. An average of 51.2 ± 1.4 urine samples were collected and analyzed for each female from time of conception to parturition and for two weeks thereafter. Data were aligned to day of parturition and pooled by week for presentation (except for LH, which is presented day by day).

Pregnant females demonstrated a continuous and significant increase in body weight from week-7 until parturition, as compared to baseline values at week-15; a sharp decline in body weight was observed within one week post-partum ($F_{20,123} = 4.38$; $p < 0.0001$; Fig. 1).

Urinary E_2 showed significant variation over time ($F_{20,105} = 4.37$; $p < 0.0001$); concentrations increased above baseline levels through mid pregnancy (weeks-9 to -6; $p < 0.05$) and showed a further increase during late pregnancy (week-5 to parturition; $p < 0.05$). After parturition, the levels exhibited a significant decline in each of weeks 1 and 2 ($p < 0.05$) (Fig. 2) prior to returning to baseline levels. These patterns were paralleled by similar significant changes in the excretion of urinary EC metabolites ($F_{20,118} = 3.22$; $p < 0.0001$, Fig. 3), but the concentrations of these metabolites rose above baseline levels only in late pregnancy from weeks-5 to -1, already falling to baseline levels at parturition ($p < 0.05$).

Urinary P_4 also varied with pregnancy status ($F_{20,104} = 1.93$; $p < 0.01$, Fig. 4), with levels showing a significant rise from week-8 to parturition followed by a decline to baseline after the pups' birth ($p < 0.05$). As with estrogens, patterns of changes in urinary PdG metabolites also varied across weeks relative to parturition ($F_{20,117} = 2.26$; $p < 0.003$, Fig. 5); however, significant increases in these hormonal metabolites were already detected in early pregnancy at week-11 (5 weeks after fertilization), followed by a significant drop to baseline at parturition (week 0) and increase again at weeks 1 and 2 post-partum ($p < 0.05$). Comparatively, this drop and subsequent increase in urinary progestagens at week 0 was only noted as a trend when using urinary P_4 determinations.

Correlation coefficients for estrogen and progestagen metabolite concentrations as determined by RIA vs. EIA were significant for all hormones ($p < 0.0001$, Table 1), yet the relationship was strongest for estrogens (72%). A positive and significant correlation was also detected for the variation across time of both hormonal metabolites in urine samples analyzed by both methods ($p < 0.0001$). Concentrations of urinary metabolites detected using EIA (EC and PdG) were ~30 and 10-fold-higher than those detected using RIA (E_2 and P_4), respectively.

No significant variations in urinary LH were detected in samples collected 15 days before and after parturition (Fig. 6; $F_{35,135} = 1$; $p = 0.47$); however, a strong tendency was observed indicating higher LH concentrations immediately after parturition at day 1.

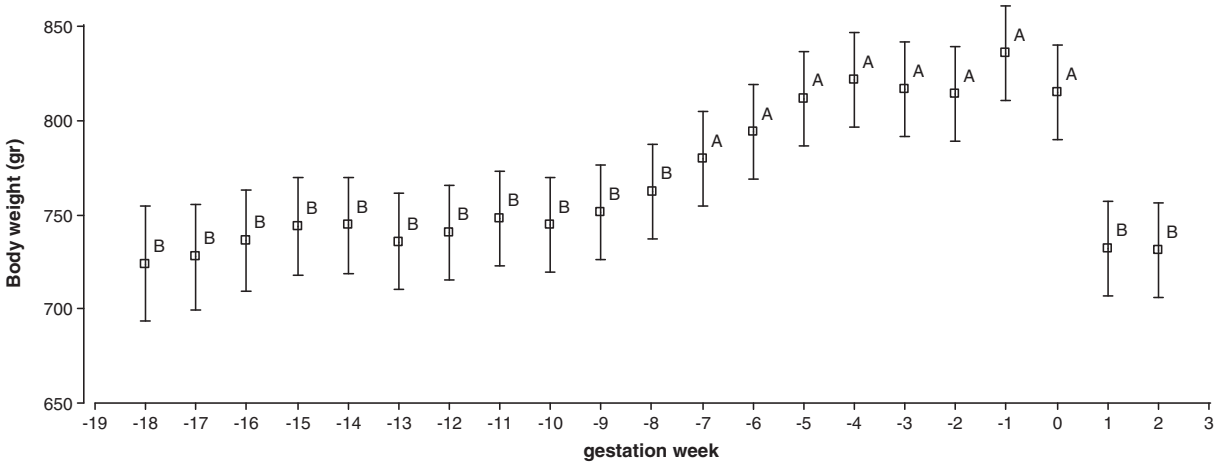

Fig. 1. Body weight variation in female domestic *Chinchilla lanigera* throughout gestation and after parturition (week 0). $n = 8$ females. (A) vs. (B) $p = 0.0001$.

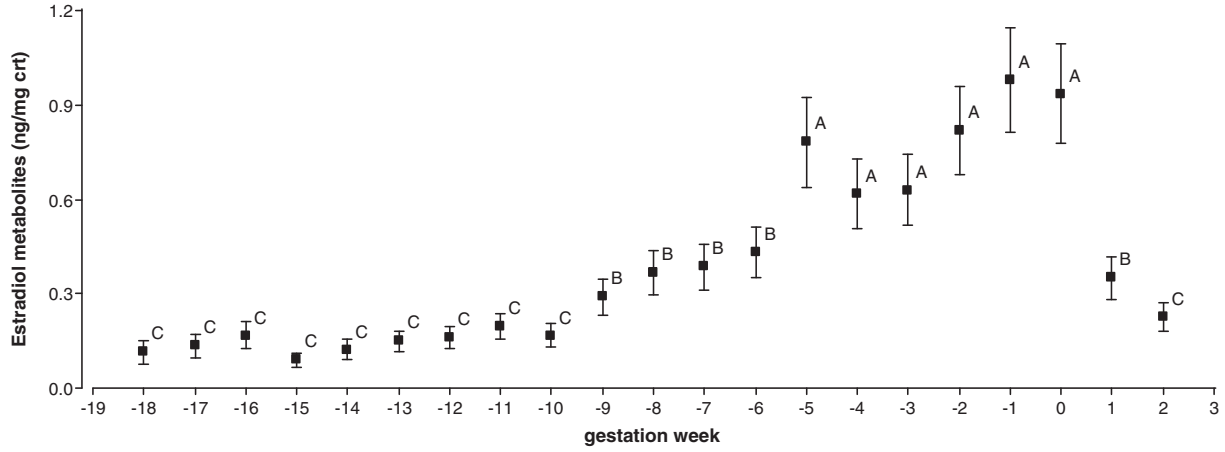

Fig. 2. Urinary estrogen metabolites variation in female domestic *Chinchilla lanigera* throughout gestation and after parturition (week 0). crt: creatinine. $n = 8$ females. (A) vs. (B) vs. (C) $p = 0.0001$.

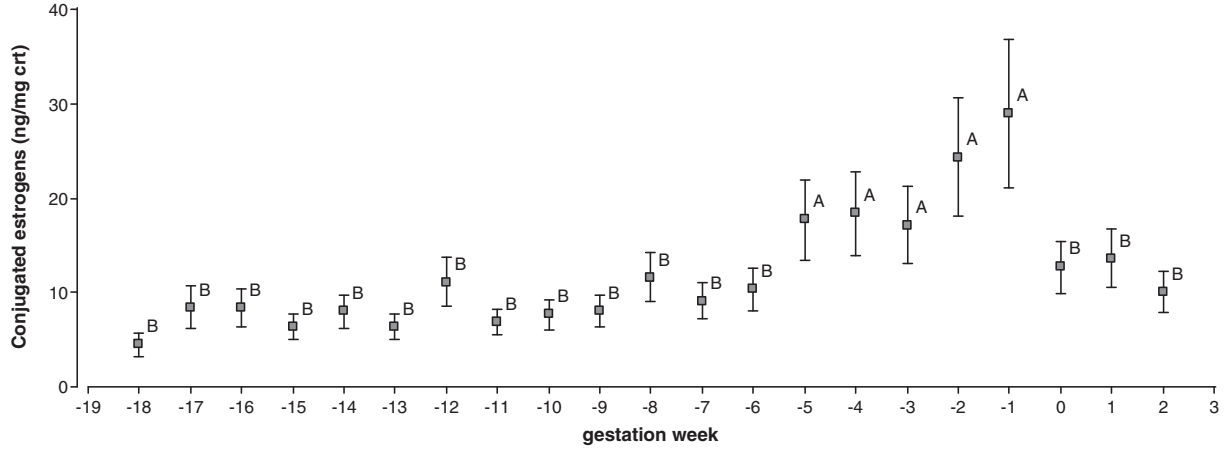

Fig. 3. Urinary conjugated estrogen metabolites variation in female domestic *Chinchilla lanigera* throughout gestation and after parturition (week 0). crt: creatinine. $n = 8$ females. (A) vs. (B) $p = 0.0001$.

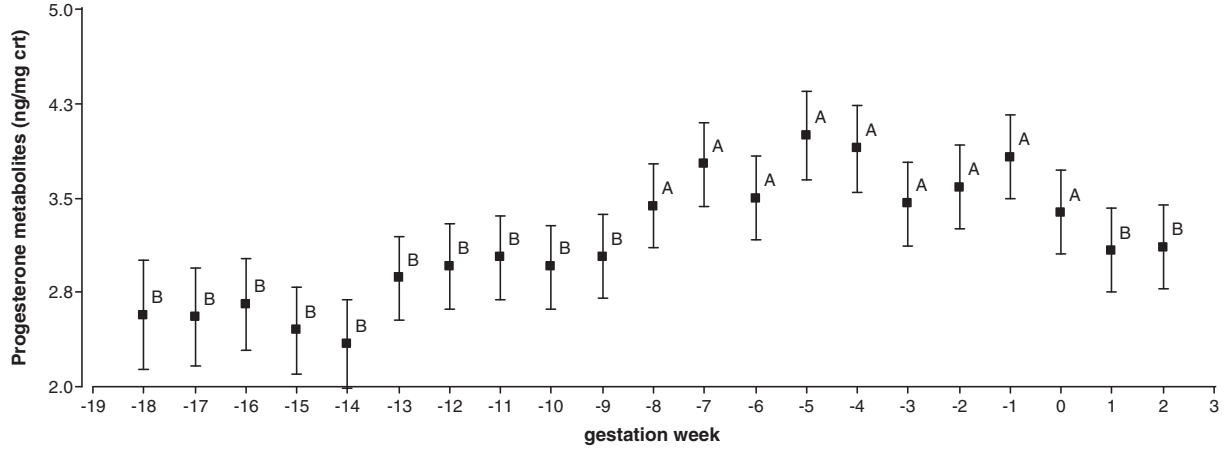

Fig. 4. Urinary progesterone metabolites variation in female domestic *Chinchilla lanigera* throughout gestation and after parturition (week 0). Data are presented as Ln of the hormone concentration. crt: creatinine. $n = 8$ females. (A) vs. (B) $p = 0.01$.

Finally, and in contrast to sex steroids, urinary cortisol metabolites did not differ significantly across pregnancy (Fig. 7; $F_{20,117} = 0.91$; $p < 0.57$); of note, we observed a tendency for higher levels of cortisol metabolites in the last third of gestation, and after parturition. If the results are analyzed by dividing the full gestation period in early, mid and late pregnancy, the latter observation is confirmed, with significantly higher cortisol metabolites concentrations in late pregnancy and the post-partum period ((in ng/mg

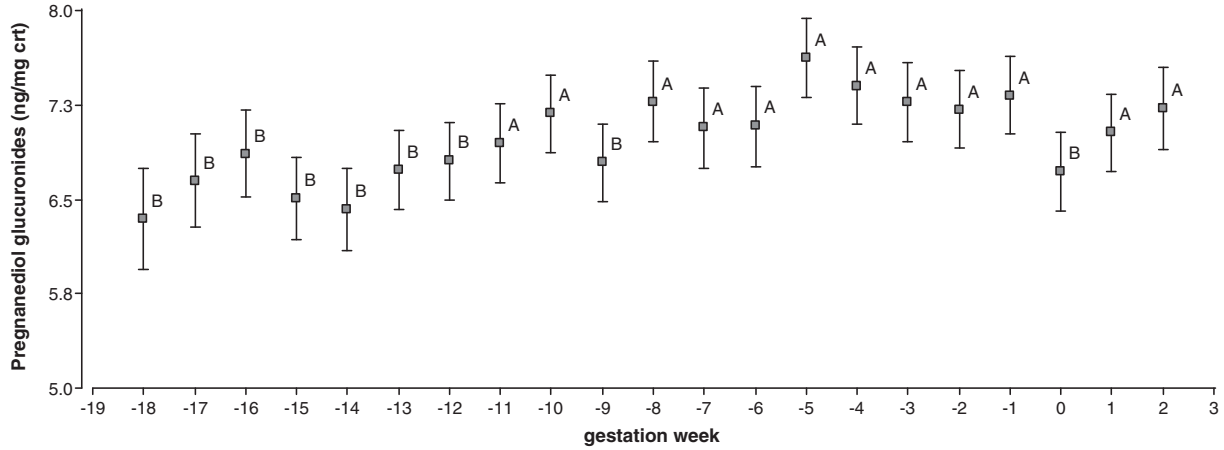

Fig. 5. Urinary pregnanediol glucuronide metabolites variation in female domestic *Chinchilla lanigera* throughout gestation and after parturition (week 0). Data are presented as Ln of the hormone concentration. crt: creatinine. $n = 8$ females. (A) vs. (B): $p = 0.003$.

Table 1
Correlation coefficients between urine samples from pre and post-partum female chinchilla ovarian steroid metabolites, analyzed using radioimmunoassay and enzyme immunoassay ($n = 8$ females).

Variable 1	Variable 2	Pearson	*p*-Value
EC	E_2	0.72	<0.0001
P_4	PdG	0.69	<0.0001
EC	PdG	0.58	<0.0001
E_2	P_4	0.69	<0.0001

crt, Ln transformed) early: 4.4 ± 0.1, mid: 4.7 ± 0.1, late: 5.1 ± 0.1, post-partum: 5.0 ± 0.1; $F_{3,134} = 5.31$; $p < 0.001$).

3.2. In vivo conception rates at post-partum estrus

Results obtained in the second experiment in commercial breeding farms regarding *in vivo* conception rates at post-partum estrus are shown in Table 2. According to the Chi-square test, no significant differences were observed in the percentages of pregnant females in any of the groups ($p = 0.16$). However, there was a tendency towards a higher percentage of fertilized females being observed on the first and third day after parturition.

The retrospective analysis of consecutive births in females maintained in breeding farm conditions showed that gestation length between first and second litter was 112.3 ± 0.16 days ($n = 217$ females). The estimated day of fertilization at post-partum estrus was 1.56 ± 0.07 days.

4. Discussion

To our knowledge, the present study is the first to fully evaluate body weight concurrently with both ovarian and adrenal steroid patterns throughout pregnancy and after whelping in farmed chinchillas and, hence, expand our knowledge of the reproductive physiology of this species.

Implementation of non-invasive hormone monitoring techniques has been previously attempted in chinchillas (Busso et al., 2007, 2005b). In female chinchillas, initial work on steroid hormone assessments focused on the excretion of estradiol and progesterone (Busso et al., 2007). While these measures allowed for the identification of significant reproductive events, the study also demonstrated that circulating E_2 and P_4 are heavily metabolized to conjugated forms prior to excretion predominately via the urinary route (>70%) (Busso et al., 2007). EC and PdG assay validations reported in the present study demonstrate that the polyclonal antisera are immunoreactive with some of the major excretory metabolites of sex steroids in female chinchillas. This was evidenced by the increased immunoreactivity detected using the EC and PdG assays compared to the E_2 and P_4 assays (30- and

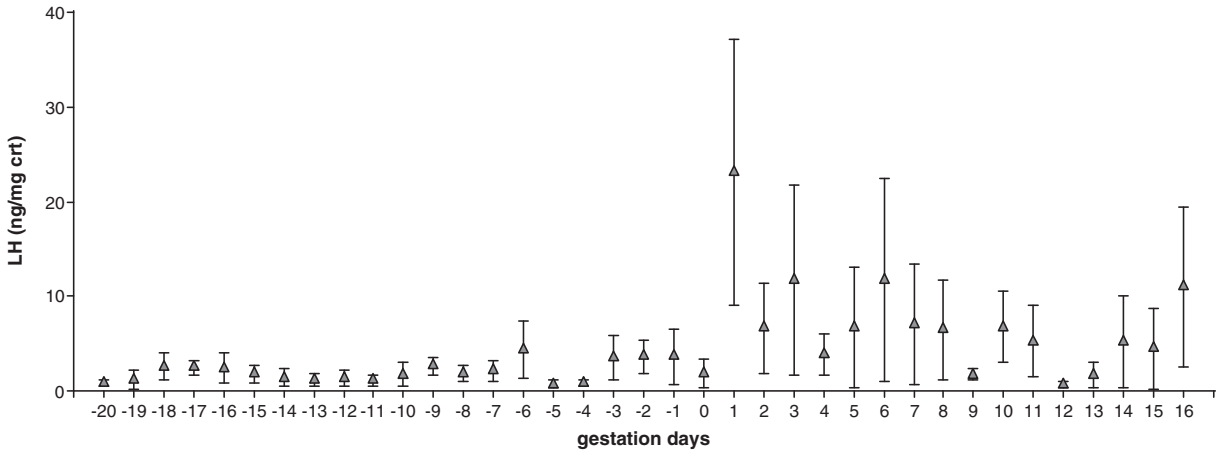

Fig. 6. Urinary LH metabolites variation in female domestic *Chinchilla lanigera* before and after parturition (day 0). Data are presented as Ln of the hormone concentration. crt: creatinine. $n = 8$ females.

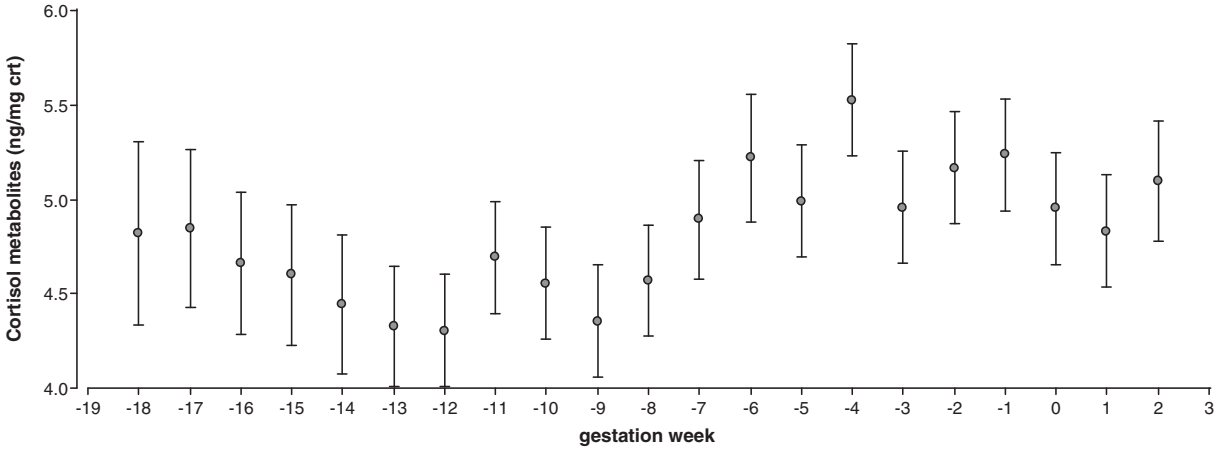

Fig. 7. Urinary cortisol metabolites variation in female domestic *Chinchilla lanigera* throughout gestation and after parturition (week 0). Data are presented as Ln of the hormone concentration. crt: creatinine. $n = 8$ females.

Table 2
In vivo conception rates after post-partum estrus in commercial chinchilla breeding farms.

Experimental group	Number of females	Pregnant females at post-partum estrus (%)
Control	18	44.4
Group 1 (24 h)	18	22.2
Group 2 (48 h)	11	18.2
Group 3 (72 h)	11	27.3
Group 4 (96 h)	16	6.3
Group 5 (120 h)	12	16.7

A total of 86 females maintained at five commercial farms were used in this study. Data are expressed as mean percentages. In parenthesis: hours after parturition when the male is allowed to mate for 24 h.

10-fold-higher, respectively) and correlation between paired EIA and RIA hormone values. The data reported in this study confirm the efficacy of the EC, PdG and LH assays to measure biologically significant changes in these excreted hormones and metabolites in chinchilla urine.

Chinchilla pregnancies, and those of other rodents belonging to the suborder Hystricomorpha, are long in contrast to Myomorpha rodent pregnancies. The range of the chinchilla's gestation is 105–115 days (Spotorno et al., 2004). According to our results, it is possible to detect pregnancy in the chinchilla 8 weeks after fertilization (~50% of the total gestation length) using body weight measures. This finding is consistent with previous validation studies from our group (Busso et al., 2007) and earlier reports suggesting that the long pregnancies of these rodents are mainly due to the extremely slow rate of fetal growth, particularly in the early stages of pregnancy (Roberts, 1971). As described by Roberts (1971), early development in chinchillas is more concerned with establishment of the placenta than with the formation of the embryo.

With respect to the endocrine patterns observed throughout pregnancy and the post-partum period, parturition was associated with dramatic reductions in urinary concentrations of sex steroids (especially progestagens), and just as in any other species, these reductions in hormone concentrations reflect the importance of the placenta in regulating hormone concentrations in pregnant chinchillas. The results of Tam's studies (Tam, 1971) indicate that the interstitial tissue of the chinchilla ovary was capable of synthesizing progesterone and estrogens as major products, and play an important role in the maintenance of gestation through its ability to produce both steroid hormones (Gromadzka-Osrttowska et al., 1985).

A few limited and contradictory earlier studies on pregnant chinchillas suggested that plasma progesterone changed from high values in early (days 9–29) and latter (days 70–80) stages of pregnancy, to very low values by days 40–60 or 90, respectively (Gromadzka-Osrttowska et al., 1985). Conversely, Tam (1970) reported that during the first 40 days of pregnancy, plasma progesterone levels did not exhibit any relevant changes, but from day 60 onwards, total hormone content reached a constant high value. This finding is in accordance with a recent study from our group validating the use of non-invasive progesterone and estrogen metabolite monitoring in chinchilla urine and feces (Busso et al., 2007) from samples collected from pregnant females at weeks-15, -11, -7, -3 and 1 after birth and assayed to measure immunoreactive E_2 and P_4 excretions. Although the sampling regimen was quite limited, a significant positive correlation was found between progestagens, estrogens and body weight during pregnancy.

Results obtained in the present work corroborate those preliminary findings of a slow but steady increase of progestagen concentrations throughout pregnancy. Urinary P_4 and PdG metabolites increased in early pregnancy between weeks-8 and -11, respectively, to parturition. This suggests that a possible confirmation of pregnancy, based on analysis of weekly urinary samples, could be feasible as early as 5 weeks after mating, which coincides with the establishment of the chorioallantoic placenta, ~30 days after mating (Spotorno et al., 2004). However, pregnancy depends also on a progressive increase in progesterone produced by a relatively high number of accessory corpora lutea and interstitial tissue (Tam, 1974). The formation of accessory corpora lutea is considered as important for supplementing progesterone during pregnancy in hystricomorph rodents such as the chinchilla, agouti (*Dasyprocta* sp.), acouchi (*Myoprocta pratti*), mountain viscacha (*Lagidium boxi*) and Canadian porcupine (*Erethizon dorsatum*) (Tam, 1974; Weir and Rowlands, 1974). As described by Weir and Rowlands (1974), the necessity for the development of excess luteal tissue in these species is apparently related to the long gestation period, throughout which the primary corpora lutea are conspicuous for the first part of gestation and then the ovary becomes cluttered by histologically identical corpora lutea. Therefore, a more clear and concise definition of pregnancy using urinary progestagen determinations would be possible 8 weeks following fertilization.

In our study, progestagen metabolites at parturition time (week 0) were found to decline slightly (yet significantly in the case of

PdG). In some mammals and other rodents, progesterone withdrawal (among other factors) precedes parturition and is the consequence of a shift in steroidogenesis that favors estrogen production at the expense of progesterone, with the associated changes from uterine quiescence to activation (for reviews see Thornburn et al., 1977; Jenkin and Young, 2004; Zakar and Hertelendy, 2007).

After the pups' births, PdG concentrations rose again whereas P_4 concentrations slightly decreased. Firstly, we should mention that these differences could be associated with the use of different assay systems in the present work. As described earlier in this discussion, previous studies in chinchilla have shown that antibodies specific for conjugated metabolites are more effective for assessing urinary progestagens in the chinchilla. Secondly, it is well known that chinchillas have a post-partum estrus at which ovulation occurs and new corpora lutea are formed. The post-partum ovary contains both the old corpora lutea of pregnancy and the new ones of the cycle, different in size and histological appearance (Weir, 1966; Tam, 1971). The somewhat elevated concentrations of progestagens in the post-partum period could be associated to corpus luteum activity following post-partum ovulation. In rats for example, Long and Evans (1922) observed that the ovaries of lactating rats contained two sets of corpora lutea; one remaining from gestation and the other forming after postpartum ovulation. Therefore, since the corpora lutea during gestation do not undergo rapid structural luteolysis, their contribution to ovarian progesterone secretion could be involved in the expression of postpartum estrus.

With respect to urinary estrogens, E_2 showed rising levels throughout mid- and late pregnancy (weeks-9 to -6 and a further increase at week-5 to parturition) and decreased after parturition, returning to baseline levels two weeks thereafter. EC metabolites on the other hand, increased only in late pregnancy, falling to baseline levels at parturition. Again, we cannot rule out the possibility that differences in assay methodology and antibody specificities may also account for these slight differences.

Elevated concentrations of estrogens during the last days of pregnancy may be due to either increased placental or ovarian estrogen production, or may result from the adrenal pathway where ACTH-stimulated cortisol promotes the synthesis of enzymes that convert progesterone to estrogens (Senger, 1997).

Estrogens generally have opposing effects to progesterone, favoring the synthesis of contractile proteins in myometrial cells and enhancing electrical coupling and the expression of oxytocin receptors, leading to well propagated, coordinated contractions (Jenkin and Young, 2004). Besides initiating parturition, the rise in estrogen secretion may play a role in the post-partum ovulation which is known to occur in other rodents such as rats and guinea-pigs (Greenwald and Rothchild, 1968). In rats for example, the post-partum increase in plasma E_2 levels stimulates release of gonadotropin releasing hormone from the hypothalamus, which in turn triggers the ovulatory luteinizing hormone surge by the pituitary gland. Additionally, female sexual behavior is induced by the sequential action of E_2 and P_4. E_2 prepares the neural substrates for lordosis, and P_4 triggers the onset and increases the intensity of this behavior (Carrillo-Martínez et al., 2011). According to the E_2 levels observed in the present work, it is quite likely that a similar physiological process occurs in the female chinchilla at post-partum estrus. Urinary E_2 remained elevated throughout the first week post-partum prior to returning to baseline levels.

Overall cortisol metabolite levels in pregnant females were observed to be relatively constant throughout pregnancy with a strong tendency for higher levels in the last third of gestation and after the pups' birth. In this respect, we should consider that the timely birth of a developmentally mature fetus requires a mechanism that synchronizes fetal maturation with the maternal mechanisms that affect the birth. The synchronizing factor has been shown to be the glucocorticoids secreted by the fetal adrenal cortex. In species in which the placenta is the principal source of progesterone for pregnancy maintenance, the increase in glucocorticoid not only directly accelerates the maturation of the fetus (Liggins and Thorburn, 1994), but also induces the expression of placental steroidogenic enzymes which redirect the steroidogenic pathway to favor the secretion of estrogens at the expense of progesterone; the resultant increase in the estrogen/progesterone ratio activates myometrial activity and labor (Jenkin and Young, 2004). After parturition, maternal glucocorticoids also play important roles in the process of milk secretion and lactogenesis (Chida et al., 2011). Although more evidence is needed, it is very likely that the chinchilla presents a similar physiological response. Also, the slightly increased cortisol levels after the pups' birth could be attributed to the behavioral mechanisms associated with the conflict between simultaneous demands of copulation and litter care at post-partum estrus.

The availability of an accurate and consistent detection for timing the LH surge in any species is critical for developing a reliable indicator of approaching ovulation. Since the pre-ovulatory estrogen peak is temporally broad-based and variable, this measure has limited use for pinpointing the time of ovulation. Conversely, urinary LH concentrations are basal until the pre-ovulatory LH surge associated with impending ovulation.

In the present work, a strong trend was observed indicating higher LH concentrations in chinchilla urine immediately after parturition (day 1). The lack of significance in this result, however, could be attributed to individual variations in the timing of the LH peak. Furthermore, this result is in agreement with observations from breeding farms, where using indirect evidence (retrospective database analyses), we can suggest that fertilization at post-partum estrus occurred after ~48 h after the litter birth.

Finally, with respect to the *in vivo* conception rates, it is interesting to note that only ~45 % of the control females allocated in these experimental conditions conceived and resulted in a positive pregnancy at post-partum estrus. However, no conclusions could be drawn with the results of this experiment due to the lack of significant differences in the percentages of females that resulted pregnant at post-partum mating in the different experimental groups. The analysis of the retrospective breeding data showed that gestation length between first and second litter in the studies females was ~112 days, and that the females that resulted in a second pregnancy after mating did so ~ 1.5 days after parturition. These data are in agreement with the LH peak detected at day 1 after the litter birth.

5. Conclusion

Urinary steroid hormone patterns of estrogen and progestagen metabolites provided valuable information on endocrine events during pregnancy and after parturition in the chinchilla. Results presented in this study enhance our understanding of natural reproductive dynamics and support empirical observations from breeders that post-partum ovulation occurs 48 h after parturition in this captive females. Non-invasive hormone monitoring techniques are a useful tool for understanding the reproductive biology of novel species and are an important first step in the development of assisted reproductive techniques.

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