Enzymeimmunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination

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ELISA measurements of 17 β -oestradiol, testosterone and progesterone were determined for urine samples collected non-invasively from female mice. Initial samples were collected during 5 successive days while mature female mice were isolated and cyclic. Subsequently, female mice were inseminated and additional urine samples were collected during days 2–6 after observation of copulatory plugs. Measurements of oestradiol and testosterone showed variance over days within individuals and did not significantly differ in measurements taken before or after

insemination. Progesterone concentrations were significantly higher after insemination compared with before mating. In a second sample of inseminated females, urinary progesterone was measured during days 2–18 of pregnancy. Most females showed high urinary progesterone up to day 10 of pregnancy and lower concentrations during the remainder of gestation. These results indicate that urinary progesterone reflects established systemic increases of this hormone during pregnancy.

Introduction

Non-invasive methods have been developed for measuring steroids in excretions of mice, permitting repeated measurements that give profiles of individuals over time and in different conditions (Muir *et al.*, 2001; Vella and deCatzanaro, 2001). Repeated blood sampling for measurements of steroids can be impractical in small species, as sampling can interfere with subsequent measurement and may require the animal to be killed. Studies over time are thus not possible unless indirect means or cross-sectional multi-subject designs are used and these can obscure individual differences. Enzyme-linked immunosorbent assays (ELISA), previously applied to larger mammals in field and zoo studies, (for example, see Carroll *et al.*, 1990; Munro *et al.*, 1991; Brown *et al.*, 1994; Graham *et al.*, 1995), have been adapted for use in mice. Muir *et al.* (2001) presented validations of methods for oestradiol, testosterone and oestrone conjugates for excretions from laboratory mice. The results indicate the substantial presence of unconjugated oestradiol and testosterone in urine and faeces of both male and female mice, but oestrone-conjugate concentrations were relatively low in female mice and undetectable in male mice. Vella and deCatzanaro (2001) measured systematic declines of oes-

tradiol and testosterone in male urine during the initial weeks after castration.

The present study was designed to examine steroid concentrations in urine during pregnancy. The first study focused on the concentrations of steroids during early pregnancy at about the time of intrauterine implantation of fertilized ova and compared these values with concentrations before insemination. In addition to measurements of oestradiol and testosterone, an assay of progesterone was validated and this steroid was measured. Individual differences in steroid dynamics during early gestation are of interest due to the sensitivity of implantation to steroid variations and the experience of the female. Early pregnancy is vulnerable to diverse environmental and social events (for example, see Hsu, 1948; Bruce, 1960; Wiebold *et al.*, 1986; deCatzanaro, 1988; deCatzanaro and MacNiven, 1992; deCatzanaro *et al.*, 1996). Although oestrogen activity is clearly important for the induction of oestrus (Pfaff, 1980) and preparation of the uterus for implantation (Harper, 1992), small increases above optimal circulating concentrations can disrupt intrauterine implantation (Harper, 1969; deCatzanaro *et al.*, 1991; 2001), whereas exogenous oestrogen antibodies can reduce vulnerability of implantation to factors such as restraint stress and exposure to novel males (deCatzanaro *et al.*, 1994, 1995). Exogenous androgens can also disrupt implantation, probably as a result of metabolism to oestrogens (Harper, 1969;

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deCatanzaro *et al.*, 1991, 2001). Progesterone is also clearly of interest given the well-known dependency of gestation upon the actions of this steroid (for example, see Runner, 1959; Pepe and Rothchild, 1974; Barkley *et al.*, 1979). In the second study, individual profiles of progesterone were determined throughout gestation.

Materials and Methods

Animals

CF-1 strain mice were bred from stock originally obtained from the Charles River Breeding Farms (La Prairie, Quebec). They were weaned at 30 days of age, and then housed in groups of four or five in standard polypropylene cages measuring 28 cm × 16 cm × 11 cm (height) with wire bar tops allowing constant access to food and water. Female mice remained in these groups before the start of the experiment and were sexually inexperienced. Male mice were housed individually after weaning, were sexually experienced and were of proven fertility, and were 6–10 months of age when used for insemination. The animal colony was maintained at 21°C with a reversed 14 h:10 h light:dark photoperiod.

This research was approved by the Animal Research Ethics Board of McMaster University, conforming to the standards of the Canadian Council on Animal Care.

Urine sampling before insemination

At approximately day 120 of age, 25 female mice were each isolated in a clear Plexiglas apparatus, measuring 30 cm × 21 cm × 13 cm (height), with a stainless steel wire-grid floor with open squares measuring 0.5 cm. The floor was raised approximately 1 cm above a clean flat stainless steel surface. Each animal had continuous access to food and water. During the first 4 days in the apparatus, female mice were completely undisturbed to allow adaptation to isolation and the apparatus. On day 5, approximately 1 h after commencement of the dark phase of the photoperiod, urine samples were collected from each female. This procedure was repeated each day for 5 successive days. Urine samples were obtained without handling the mice by lifting the apparatus away from the stainless steel floor, and then aspirating urine from pools left by the mice with a 1 cc syringe with a 26-gauge needle. Approximately 500 µl of urine was collected per mouse per day. Care was taken to ensure that urine samples were not contaminated with faeces. All samples were stored after collection in coded scintillation vials at –20°C.

Insemination, subsequent collection of urinary samples and parturition measurements

Three days after the last collection of urine before insemination, each female was transferred to the home cage of a male mouse. The hindquarters of each female

were inspected on three occasions each day (1, 5 and 9 h after commencement of the dark phase of the photoperiod) for copulatory plugs. Plugs were found for eight females on day 1, four females on day 2, three females on day 3 and five females day 4 after housing with the male. Immediately after detection of a copulatory plug, each female was returned to the urine collection apparatus. One other female mouse was eliminated from the experiment due to poor health; four other female mice were eliminated because copulatory plugs were not detected. Accordingly, 20 inseminated female mice were each in a urine collection apparatus. Five additional daily collections of urine were conducted for each inseminated female on days 2–6 after detection of the copulatory plug, using the methods described for collections before insemination. After the fifth collection of urine after insemination, each female was removed from the collection apparatus, housed individually in a standard mouse cage with nesting material and left undisturbed until pregnancy outcome measurements were taken. Commencing on day 18 and continuing until day 25 after detection of the copulatory plug, each female was examined on three occasions each day for occurrence of birth. The number of live pups, the number of stillbirths and cannibalizations were recorded.

Urine progesterone throughout gestation

Given the results from the first study indicating that urine progesterone increased in early pregnancy, a second study was conducted to determine the concentration of urinary progesterone throughout pregnancy. Six female mice were mated to males as described above and then placed at approximately 24 h after detection of a copulatory plug in urine collection cages similar to those described above, except that a Teflon-coated collection surface was used to improve urine sampling. Samples were collected from each female each day at approximately 1 h after the start of the dark phase of the photoperiod, beginning on day 2 and ending on day 18 after the detection of the copulatory plug.

Assay procedures

ELISA procedures generally followed those described for other mammals (Munro *et al.*, 1991); validations for this laboratory for adult male and female mice are reported by Muir *et al.* (2001). Coefficients of variation were determined by the methods described by Rodbard and Lewald (1974). For oestradiol, the interplate coefficient of variation was 8.4% at 30% bound and 4.1% at 70% bound, and the intraplate coefficient of variation was 8.7%. For testosterone, the interplate coefficients of variation were 6.7% at 30% bound and 3.4% at 70% bound, and the intraplate coefficient of variation was 7.1%. For progesterone, the interplate coefficients of variation were 9.7% at 30% bound and 4.1% at

70% bound, and the intraplate coefficient of variation was 7.4%. Creatinine, 17 β -oestradiol, testosterone and progesterone were obtained from Sigma Chemical Co. (St Louis, MO) Antibodies to 17 β -oestradiol, testosterone and progesterone and corresponding horseradish peroxidase conjugates were obtained from the Department of Population Health and Reproduction at the University of California (Davis, CA). Crossreactivities according to the criteria of Abraham (1969) for anti-oestradiol are: oestradiol 100%, oestrone 3.3%, progesterone 0.8%, testosterone 1.0%, androstenedione 1.0% and all other tested steroids <0.1%. Crossreactivities for anti-testosterone are: testosterone 100.0%, 5 α -dihydrotestosterone 57.4%, androstenedione 0.27%, and androsterone, dehydroepiandrosterone (DHEA), cholesterol, oestradiol, progesterone and pregnenolone <0.05%. Crossreactivities for anti-progesterone are: progesterone 100.0%, 11 α -hydroxyprogesterone 45.2%, 5 α -pregnen-3,20-dione 18.6%, 17 α -hydroxyprogesterone 0.38%, 20 α -hydroxyprogesterone 0.13%, 20 β -hydroxyprogesterone 0.13%, pregnanediol <0.001%, pregnenolone 0.12%, oestradiol <0.001% and oestrone <0.04%.

The assays were carried out on Nunc Maxisorb plates which were first coated with 50 μ l of antibody stock diluted at 1:10000 in a coating buffer (50 mmol bicarbonate buffer l⁻¹, pH 9.6) and stored for 12–14 h at 4°C. Wash solution (0.15 mol NaCl l⁻¹ containing 0.5 ml of Tween 20 l⁻¹) was added to each well to rinse away any unbound antibody and then 50 μ l phosphate buffer per well was added. The plates were incubated at room temperature (21°C) for 2 h for oestradiol determination, 30 min for testosterone determination and 1 h for progesterone determination before adding standards, samples or controls. For oestradiol determination, urine samples were diluted 1:8 in phosphate buffer before they were added to the plate. For testosterone or progesterone assays, urine was diluted 1:4 each in phosphate buffer. For each hormone, two quality control urine samples at 30 and 70% binding (the low and high ends of the sensitive range of the standard curve) were prepared. For all assays, 50 μ l oestradiol, testosterone, or progesterone horseradish peroxidase was added to each well, with 20 μ l of standard, sample, or control for oestradiol or 50 μ l of standard, sample, or control for testosterone or progesterone. The plates were incubated for 2 h at room temperature. Subsequently, the plates were washed and 100 μ l of a substrate solution of citrate buffer, H₂O₂ and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) were added to each well and the plates were covered and incubated while shaking at room temperature for 30–60 min. The plates were then read with a single filter at 405 nm on the microplate reader (Bio-Tek Instruments Inc. EL 312E). Blank absorbance was subtracted from each reading to account for non-specific binding.

In all assays, absorbances were obtained, standard curves were generated, a regression line was fit to the

sensitive range of the standard curve (typically 40–60% binding) and samples were interpolated into the equation to get a value in pg per well. Validations for assays of oestradiol and testosterone were provided by Muir *et al.* (2001). Validations for progesterone were subsequently conducted and reported here. A test for parallelism was conducted to determine whether urine samples perform immunologically in a similar manner to steroid standards. A serial dilution of samples was compared with a standard curve plotted against logarithmically transformed doses. This test indicates whether the steroid is present in samples in measurable quantities, and whether samples react with the antibodies in a predictable manner. In addition, progesterone concentration in urine samples from ovariectomized and intact females were compared. Twelve females were ovariectomized under sodium pentobarbital anaesthesia and Xylocaine at the site of incision. After approximately 1 month, samples of their urine were collected and pooled. Samples of urine from 24 group-housed intact females were also collected and pooled.

On account of variations in fluid intake and output, concentration of urine in experimental samples was adjusted for creatinine. Standard creatinine values of 100.0, 50.0, 25.0, 12.5, 6.25 and 3.12 μ g ml⁻¹ were used, and distilled water was set at zero. All urine samples are diluted 1:50 urine:phosphate buffer (0.1 mol l⁻¹ sodium phosphate buffer, pH 7.0 containing 8.7 g of NaCl and 1 g of BSA per litre). Dynatech Immulon flat bottom plates were used and 50 μ l per well of standard was added together with 50 μ l distilled water, 50 μ l 0.75 mol NaOH l⁻¹ and 50 μ l 0.4 mol picric acid l⁻¹. The plate was then shaken and incubated at room temperature for 30 min. The plate was measured for absorbance on a plate reader with a single filter at 490 nm. Standard curves were generated; regression lines were fit; and the regression equation was applied to the absorbancy for each sample. Steroid measurements were adjusted for creatinine by dividing the value obtained by the measurement of creatinine per ml of urine for the particular sample.

Results

The results of a test for parallelism for progesterone that compare serially diluted samples and standards are shown (Fig. 1). A test for parallelism was performed on these two curves (Pedhazur, 1973); application of the regression equation derived from the standard curve fit the urine data as well as did the regression equation predicting urine itself, with almost identical *R*² values (0.8501 versus 0.8503) for a quadratic fit. Progesterone was able to displace horseradish peroxidase-conjugate binding from 100% down to 17% when samples were tested in serial dilutions from 1:512 to 1:1 (urine:buffer). Measurements of urinary progesterone yielded a value

Fig. 1. Serially diluted pooled mouse urine samples binding to antibody in parallel with serially diluted progesterone standards. The abscissa applies only to the standards. ◇: standard; ■: urine.

of 16.8 ng mg⁻¹ creatinine for pooled intact females and 4.0 ng mg⁻¹ creatinine for females ovariectomized 1 month previously; a ratio of 4:1 or greater was sustained in comparisons of progressive dilutions of the urine. Sensitivity of the progesterone assay was determined by the least amount of hormone that could be distinguished from zero concentration of standard, as calculated from 95% confidence limits at the zero point of the standard curve. The assay had a range of 2.4–2500.0 pg per well with a minimum sensitivity of 4.8 pg per well (50 µl). Recovery curves were generated to assess procedural losses of hormone. Steroid standards were added to pooled urine samples at three different concentrations and extracted for measurement. The average recovery for spiked urine samples was 95.0 ± 11.0%.

The mean (± SE) oestradiol concentration in the five measurements before insemination and during the five measurements on days 2–6 after insemination, including data in both cases from the 20 subjects that had copulatory plugs, is shown (Fig. 2). The corresponding data for measurements of testosterone and progesterone are shown (Figs 3 and 4). The strongest and most obvious effect was a higher overall concentration of progesterone in the urine of female mice after insemination as opposed to before insemination. ANOVA was conducted on each steroid measured for the 20 mice with copulatory plugs, treating day of measurement and the before versus

Fig. 2. Mean (± SE) values for oestradiol in (a) the five successive daily urine measurements before insemination and (b) the five measurements on days 2–6 after insemination, for all mice in which copulatory plugs were observed.

Fig. 3. Mean (± SE) values for testosterone in (a) the five successive daily urine measurements before insemination and (b) the five measurements on days 2–6 after insemination, for all mice in which copulatory plugs were observed.

Fig. 4. Mean (± SE) values for progesterone in (a) the five successive daily urine measurements before insemination and (b) the five measurements on days 2–6 after insemination, for all mice in which copulatory plugs were observed.

Table 1. Pearson product-moment correlations between the number of mice born and steroid measurements on 5 successive days before insemination and 5 successive days after insemination (days 2–6 after detection of a copulatory plug) in mice

	Number of mice born	After day 5	After day 4	After day 3	After day 2	After day 1	Before day 5	Before day 4	Before day 3	Before day 2
Oestradiol										
Before day 1	+0.08	+0.03	+0.28	+0.25	+0.14	+0.36	+0.30	+0.29	−0.28	+0.23
Before day 2	+0.01	−0.17	+0.33	−0.14	+0.07	+0.54	+0.55	+0.06	+0.35	
Before day 3	−0.18	−0.00	+0.06	−0.33	+0.21	+0.06	+0.24	−0.05		
Before day 4	+0.20	+0.03	+0.43	+0.41	+0.07	+0.29	+0.45			
Before day 5	+0.23	−0.19	+0.30	−0.07	+0.00	+0.75*				
After day 1	+0.33	−0.06	+0.26	+0.03	−0.24					
After day 2	−0.59*	−0.07	+0.25	+0.12						
After day 3	−0.21	+0.09	+0.53							
After day 4	−0.21	+0.33								
After day 5	+0.12									
Testosterone										
Before day 1	+0.04	+0.22	+0.51	+0.51	+0.15	+0.50	+0.64*	+0.54	+0.25	+0.59*
Before day 2	−0.01	+0.35	+0.42	+0.36	+0.35	+0.55	+0.54	+0.55	+0.66*	
Before day 3	+0.05	+0.51	+0.58*	+0.54	+0.33	+0.69*	+0.39	+0.75*		
Before day 4	+0.08	+0.43	+0.73*	+0.79*	+0.27	+0.70*	+0.61*			
Before day 5	+0.20	+0.08	+0.61*	+0.54	+0.08	+0.55				
After day 1	+0.18	+0.33	+0.49	+0.79*	+0.31					
After day 2	−0.33	−0.09	+0.08	+0.25						
After day 3	+0.03	+0.35	+0.77*							
After day 4	+0.07	+0.38								
After day 5	+0.21									
Progesterone										
Before day 1	+0.31	−0.15	+0.14	+0.08	−0.11	+0.13	+0.28	+0.36	−0.11	−0.04
Before day 2	+0.13	+0.17	+0.24	+0.35	+0.44	+0.68*	+0.79*	+0.45	+0.57*	
Before day 3	+0.19	+0.47	+0.07	+0.15	+0.23	+0.51	+0.62*	+0.44		
Before day 4	+0.22	−0.08	+0.36	+0.37	+0.30	+0.45	+0.64*			
Before day 5	+0.34	+0.18	+0.40	+0.52	+0.20	+0.74*				
After day 1	+0.38	+0.14	+0.55	+0.50	+0.05					
After day 2	−0.02	+0.11	+0.16	+0.11						
After day 3	−0.05	+0.21	+0.86*							
After day 4	+0.14	+0.28								
After day 5	+0.19									

A positive sign indicates a positive correlation, whereas a negative sign indicates an inverse correlation.

*Two-tailed probability < 0.01.

after insemination factor as within-subjects repeated measures. There were no significant effects for oestradiol. For testosterone, there was a significant interaction between day of measurement and the before versus after insemination factor, $F(4,171) = 3.36$, $P = 0.011$. Multiple comparisons (Duncan's new multiple range test, $P < 0.05$) indicated that the first measurement before insemination significantly exceeded all other measurements except the measurement after the final insemination, and that the final measurement after insemination significantly exceeded the measurements from the previous 2 days. For progesterone, there was a clearly significant effect of the before versus after insemination factor, $F(1,171) = 37.33$, $P < 0.0001$, but no other significant effects.

Of 20 female mice in which copulatory plugs were detected, 16 were parturient. Eighteen of the 20 female mice were observed to have built nests during week 2 after insemination; the other two female mice were among the non-parturient females. During days 1–2 after birth, three of the parturient female mice completely cannibalized their litters and one other female cannibalized part of her litter. The mean ($\pm$ SE) number of pups among parturient females was 9.81 ± 1.01 and the mean number of pups surviving on day 3 was 8.06 ± 1.43 . Pearson product-moment correlations calculated between the number of pups born and each daily steroid measurement and among measurements within each steroid for 20 inseminated females, (zero was assigned to non-parturient females) are shown (Table 1).

Table 2. Pearson product-moment correlations among steroid measurements (oestradiol, progesterone and testosterone) on mouse urine samples before insemination and after insemination

	Oestradiol–progesterone	Oestradiol–testosterone	Testosterone–progesterone
Before day 1	+0.441	+0.823*	+0.154
Before day 2	+0.667*	+0.624*	+0.267
Before day 3	+0.377	+0.145	+0.342
Before day 4	+0.496	+0.348	+0.167
Before day 5	+0.772*	+0.728*	+0.415
After day 1	+0.650*	+0.736*	+0.504
After day 2	+0.386	+0.555	+0.229
After day 3	+0.516	+0.636*	+0.356
After day 4	+0.657*	+0.748*	+0.608*
After day 5	+0.909*	+0.888*	+0.871*

*Two-tailed probability < 0.01.

Fig. 5. Individual values of progesterone in daily urine measurements during days 2–18 of gestation for six inseminated female mice.

Correlations among repeated oestradiol measurements were generally lowest and correlations among repeated testosterone measurements were highest, indicating that oestradiol concentrations vary the most and testosterone the least over days within individuals. The number of pups born related clearly to only one steroid measurement, which was an inverse relationship to oestradiol concentrations on the second measurement after insemination. Correlations among the three steroid measurements were generally positive for measurements taken on the same day and not significant for comparisons across days; those for the same day are shown (Table 2).

Daily values of creatinine-adjusted urinary progesterone for six mice monitored from day 2 to day 18 of gestation are shown (Fig. 5). Overall values of progesterone varied among mice. Five of six mice showed a pattern

of increased progesterone from day 3 or day 4 to day 10 of gestation with substantially lower values for the remainder of gestation. One of these mice had relatively low and invariant concentrations of urinary progesterone. All female mice were parturient and had litters in the range of 8–12 live pups.

Discussion

A reliable and sensitive method for measuring variations in unconjugated oestradiol, testosterone and progesterone in mouse urine has been developed. Unlike procedures that require handling of animals and collection of blood samples, these methods permit repeated measurements and profiling of individuals over time.

Insofar as urinary measurements reflect systemic action of these hormones, this may provide a non-invasive method to relate steroid dynamics to ongoing behaviour and development.

The most significant finding in the present study was that progesterone concentrations were substantially higher after insemination than before insemination. This finding was clearly significant in the first study and was then repeated in the second study in the individual profiles of urinary progesterone at days 2–18 of gestation. In the second study, there was clearly an increase in urinary progesterone from day 3 or day 4 to day 10 after detection of the copulatory plug for five of six mice. However, results from the one subject for which the progesterone concentrations were less dynamic during pregnancy cannot be accounted for, as this mouse produced a normal litter. The increase in urinary progesterone during pregnancy is generally consistent with the well-known systemic increase in progesterone during pregnancy. Nevertheless, unlike the current results, other reports indicate increased plasma progesterone during or up to late pregnancy, reaching a peak at about days 15–17 (McCormack and Greenwald, 1974; Pepe and Rothchild, 1974; Holinka *et al.*, 1979), and similar profiles have been reported for rats (Barkley *et al.*, 1979). The apparent discrepancies between blood and urinary concentrations of progesterone in late pregnancy could reflect strain differences, differential protein binding at different stages of pregnancy or various metabolic factors that have yet to be explored. Subsequent work should focus also on pregnanediol-3 α glucuronide (*cf.* Tyler *et al.*, 1978); the ratio of conjugated and unconjugated progesterone during pregnancy potentially changes with stage of gestation.

In the measurements after insemination, progesterone concentrations did not show clear individual cycles, and there was a positive correlation over the 5 days. Variation of progesterone within individuals over the oestrous cycle might be obscured in part by sampling relative to a diurnal cycle (*cf.* Michael, 1976; Bailey, 1987); proper characterization might require multiple measurements per day over several days. Oestradiol concentrations were more dynamic, showing low intra-individual correlations over days. In additional work (D. deCatanzaro, E. Beaton, C. Muir, M. Jetha and N. Laetsch, unpublished), we have observed clear cycling of urinary oestradiol within individuals over several successive days. Cell identifications in vaginal smears have indicated that the duration of the oestrous cycle in mice is variable and idiosyncratic, averaging about 4–6 days, but often not regularly recurring within a particular individual (for example, see Nobunaga, 1973; Barkley and Bradford, 1981; DeLeon *et al.*, 1990).

The significant increase in testosterone in the fifth measurement after insemination (day 6 after detection of a copulatory plug) is consistent with the increase in plasma concentrations of this hormone that has been

reported by Barkley *et al.* (1977, 1979). Otherwise, urinary testosterone was generally stable within individuals, showing quite high correlation values among daily samples.

The number of pups born was inversely related to oestradiol concentrations on the second measurement after insemination, which was approximately 70–80 h after mating, corresponding to intrauterine implantation of fertilized ova. Although oestrogen activity is clearly important for preparing of the uterus for implantation (Harper, 1992), high plasma oestradiol can cause disintegration of the corpus luteum (Greenwald, 1964), disrupt timing of arrival of fertilized ova at the uterus (Burdick and Whitney, 1937), produce suboptimal endometrial receptivity (Suginami, 1995) and induce oestrus via actions at the ventromedial hypothalamus (Pfaff, 1980).

Direct correlation of systemic and urinary steroid measurements for mice is limited in that blood sampling is too invasive to permit repeated systemic measures and subsequent urinary analysis, preventing full analysis of time lags between blood and urinary measurements. Unconjugated hormones as measured in urine in this laboratory do reflect some well-established systemic patterns. Systematic declines in urinary testosterone are observed after castration of males (Vella and deCatanzaro, 2001). Urinary oestradiol shows dynamic variation in cyclic females. As evident in data from the current study, urinary progesterone is substantially lower in samples from ovariectomized females than in those from intact females, and significantly increased in pregnant females to concentrations above those measured before insemination.

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) awarded to D. deCatanzaro.

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Received 24 February 2003.

First decision 10 April 2003.

Revised manuscript received 30 April 2003.

Accepted 19 May 2003.