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Urinary levels of oestradiol and pregnanediol glucuronides reflect the ovarian function in the domestic dog (*Canis familiaris*)

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ABSTRACT

In many mammals, the reproductive cycle can be non-invasively monitored by measuring the concentrations of urinary steroid hormone metabolites. In domestic dogs, however, there is limited information available on the urinary excretion of oestrogens and progesterone. Oestradiol-3-glucuronide (E2G) and pregnanediol-3-glucuronide (PdG), the urinary metabolites of oestradiol-17β and progesterone, respectively, are commonly reported components of mammalian urine. In this study, we collected one to 28 daily urine samples of 43 female dogs after the onset of sanguineous vaginal discharge during 50 reproductive cycles. We developed an ELISA method for the measurement of urinary concentrations of E2G (nine cycles) and PdG (50 cycles). A linear mixed model was used to estimate the expected values and 95 % confidence intervals for the E2G and PdG concentrations. The expected urinary E2G was estimated to peak 6 days after the onset of sanguineous vaginal discharge and to decrease to one-third of the maximum concentration within a week. The estimates of the expected urinary PdG concentrations were low at the onset of sanguineous vaginal discharge and increased slowly thereafter, reaching maximum concentrations within three weeks. The similarity of the E2G and PdG models with existing literature suggested that urinary steroid metabolites could be a useful biomarker for detecting ovarian function in female dogs. Further detailed investigation is needed to enhance the suitability of E2G and PdG measurements in monitoring of oestrus. In summary, measurement of the urinary metabolites of steroid hormones is a promising non-invasive method for studying reproductive physiology in female dogs.

1. Introduction

The oestrous cycle of the domestic dog (*Canis familiaris*) consists of four stages: anoestrus, pro-oestrus, oestrus, and dioestrus. The concentration of serum oestradiol (oestradiol-17β, E2) increases during pro-oestrus, reaching a maximum concentration (45–120 pg/mL) 1–3 days before the peak of luteinising hormone (LH), which is usually described as the beginning of oestrus. After reaching the maximum value, the E2 concentration decreases rapidly to the basal level (5–10 pg/mL) (Concannon, 2011). Serum progesterone (P4) remains at a low basal level (0.2–0.5 ng/mL) during anoestrus and begins to rise just before the LH peak. The P4 concentration is

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approximately 3.9–8.5 ng/mL during ovulations occurring 2–3 days after the LH peak and reaches the highest concentration level (15–60 ng/mL) a few weeks after the LH peak. Regardless of whether the female dog is pregnant or not, the P4 level remains elevated for 50–90 days before decreasing to the basal level (Concannon, 2011; Hollinshead and Hanlon, 2019).

In humans and other mammalian species, such as bison, cow, and giant panda, hormonal changes of P4 can be monitored non-invasively via the measurement of urinary pregnanediol glucuronide (PdG) (Monfort et al., 1990; Munro et al., 1991; Kirkpatrick et al., 1992; Yang et al., 2004; Shimizu, 2005; Hama et al., 2009). In human, Goeldi's monkey and bison, urine PdG measurement can be used to determine the time of ovulation (Kirkpatrick et al., 1992; Pryce et al., 1993; Roos et al., 2015), and in Eld's deer, bison and Holstein dairy cows, elevated PdG concentrations can be interpreted as a sign of pregnancy (Monfort et al., 1990; Kirkpatrick et al., 1992; Yang et al., 2004). Measurements of urinary E2 have been used to monitor the seasonal luteal activity of the Iberian lynx and Sumatran rhinoceros (Heistermann et al., 1998; Jewgenow et al., 2009). Combined measurements of urinary E2–3-glucuronide (E2G) and PdG have been reported to be effective in monitoring the reproductive status of Sichuan golden monkeys (Muren et al., 2017).

Very little information is available on the metabolism and urinary excretion of oestrogens and P4 in domestic dogs. Most studies concerning urinary oestrogens were conducted over a half-century ago. A significant correlation exists between circulatory and excreted oestrogens (Batchelor et al., 1972). In studies in which radiolabelled oestrogens (E2 and oestrone (E1)) were administered intravenously to domestic dogs, most oestrogen metabolites were excreted in the urine as glucuronides. Oestradiol-17α and E2 were found to be the major urinary metabolites, and significant amounts of E1 and small quantities of oestriol (E3) were also found in the urine (Siegel et al., 1962; Balikian et al., 1968; Beling et al., 1975).

In the family Canidae, there is only one peer-reviewed study concerning the measurement of urinary P4 metabolites. In African wild dogs, the urinary PdG profile corresponded to the serum profile of P4 in the literature but was not considered the primary metabolite of P4 in the urine (Monfort et al., 1997). Since all the canids have similar luteal activity and circulatory secretion profiles of P4 (Concannon et al., 2009), it can be assumed that the measurement of PdG is also feasible in domestic dog urine. Besides urine sample collection being non-invasive, urine is an easy and affordable sample matrix. Instead of blood sampling or faeces collection, urine sample collection can be easily performed without presence of professionals, and usually do not require extraction methods prior analysis. The aims of this study were to develop an ELISA for the measurement of urinary E2G and PdG, and to determine the suitability of urinary E2G and PdG measurements for monitoring of ovarian function in female dogs.

2. Materials and methods

2.1. Ethics of experimentation

In this study, urine samples were collected by volunteer dog owners, and the samples were collected via the free-catch method without forcing or harming the animals. Invasive methods such as vaginal cytology and blood sampling were intentionally avoided to prioritise animal welfare and encourage owner participation. The collection of urine samples was completely non-invasive, did not cause pain, suffering, or harm to the female dogs, and did not interfere with their normal behaviour. No invasive oestrus monitoring methods were required for the study, and ethical evaluation and approval were not necessary. All dog owners actively participated in sampling and consented to the use of urine samples in this study.

2.2. Animals and sampling

The field material included urine samples collected by volunteered dog owners. The purpose was to collect urine samples from multiple dogs after the onset of sanguineous vaginal discharge. All participants were interviewed before admitting to the research to ensure they are experienced and recognised the signs of oestrus in their female dogs: all dog owners were aware of the meaning of sanguineous vaginal discharge, and according to the interviews and diary markings, they were able to recognise the oestrous behaviour in their female dogs (standing oestrus, displaying the vulva, turning the tail) and were familiar with the duration and oestrus behaviour of their female dogs' previous oestruses.

A total of 603 morning urine samples of 43 female dogs comprising 50 reproductive cycles were collected from 2012 to 2016. According to dog owners, all female dogs had been in oestrus at least once before. The group of the 43 female dogs consisted of 32 different breeds (31 purebreds and one mixed breed) with an average body weight of 21.3 kg (ranged from 3.4 kg to 64 kg) and an average age of three years 11 months (ranged from one year to eight years four months). The descriptions of the breeds, ages and weights of the female dogs included in the study are listed in Supplementary Table S.1.

The onset of sanguineous vaginal discharge was considered as Day 0. The dog owners were requested to start collecting daily urine samples at latest on Day 5 and collect at least ten urine samples until the cessation of sanguineous vaginal discharge or until the dog was mated or inseminated. The samples were asked to be collected from the first spontaneous urination in the morning. Free-catch urine samples were collected via an optional plastic cup or dish, and the collected urine was transferred into labelled polypropylene tubes with a maximum volume of 15 mL. The samples were frozen in dog owners' household freezers (at approximately −20 °C) without preservatives and kept frozen until they were transported to the laboratory at the end of the collection period. The urine samples were stored at −20 °C in the laboratory until analysis. The thawed samples were centrifuged for 5 min at 3220 X g (Eppendorf Centrifuge 5890), and the aliquoted urine supernatant was used for the assays.

The dog owners were requested to keep a diary for the signs of oestrus in their female dogs, including initiation and cessation of sanguineous vaginal discharge, and the dates of standing oestrus, mating and insemination. After the collection of urine samples, the dog owners were asked to inform the researchers of possible pregnancies, the date of birth, the number of puppies, and any other

matters occurring afterwards.

2.3. Competitive E2G ELISA

A competitive E2G ELISA was developed using commercial polyclonal rabbit E2G antibodies. The polystyrene 96-well microplates (655101; Greiner Bio-one GmbH, Frickenhausen, Germany) were coated by adding 100 µL of 1 µg/mL goat anti-rabbit IgG (611–1122; Rockland, PA, USA) in coating buffer (10 mM sodium bicarbonate buffer, pH 9.5), sealed with adhesive tape, and kept in the dark at room temperature overnight or in the refrigerator for no longer than a week. The plates were washed three times with 250 µL of washing buffer (0.05 % Tween 20 in PBS (116 mM NaCl, 10.6 mM Na_2HPO_4*$2H_2O$, 1.5 mM KH_2PO_4; pH 7.4)). Rabbit polyclonal E2G antiserum (FKA-238-E; Cosmo Bio Co., Ltd., Tokyo, Japan) was diluted 1:500 in sample buffer (0.5 % BSA (w/V; fraction V, Rockland, Gilbertsville, NY, USA) in PBS), and 100 µL was added per well. The plates were incubated at + 37 °C with sealing tape for 30 min and washed as described above. The cross-reactivities of the antibodies were reported as 100 % E2G, 104 % E2, 18 % E1–3-glucuronide, 16 % E2–3-sulphate, 11 % E1, 1 % E3 and less than 1 % with all other steroids tested (testosterone, pregnenolone, P4, cortisol, cortisone, 4-androstenedione, dehydroepiandrosterone, dihydrotestosterone). A seven-point 1:2 standard dilution series from 20 ng/mL to 0.3 ng/mL of E2 (Sigma—Aldrich, St. Louis, MO, USA) was prepared in sample buffer. Standard urine was prepared by pooling nine urine samples of anoestrous female dogs and three urine samples from sterilised female dogs, and the pool was diluted in water to a creatinine concentration of 1 mg/mL (approximately 1:2 dilution). The standard urine and urine samples were diluted 1:20 in sample buffer. Fifty microliters of E2 standards, standard urine (control wells) and urine sample dilutions, and ultrapure water (maximum binding wells) were added to each well in duplicate. Horseradish peroxidase-labelled E2G (E2G-HRP, FKA-237, Cosmo Bio Co., Ltd., Tokyo, Japan) was diluted 1:250 in sample buffer, and 50 µL was added to each well. The plates were sealed with adhesive tape and incubated for 30 min at + 37 °C. After the plates were washed, the substrate mixture was prepared just before use by mixing the TMB solution (0.5 % 3,3′,5,5′-tetramethyl benzidine (w/V, T2885, Fluka, Buchs, Switzerland) in dimethyl sulfoxide (Fisher Scientific, Loughborough, UK)) with substrate buffer (0.1 M sodium acetate trihydrate, 1.5 mM citric acid monohydrate, 0.005 % H_2O_2) at a 1:50 ratio. The substrate mixture was added at 100 µL/well, and the enzymatic reaction was stopped after 30 min by adding 50 µL of 1 M sulfuric acid (H_2SO_4, ≥98 % purity, Merck Millipore, Darmstadt, Germany) to each well. The optical density at 450 nm was measured with a microplate reader (Multiskan RC, Thermo Labsystems Ltd.).

2.4. Competitive PdG ELISA

A competitive PdG ELISA was developed using a custom polyclonal rabbit PdG antibody. The custom antibody against PdG–KLH (keyhole limpet hemocyanin) was raised in rabbits, and total IgG was purified in a protein A column by the manufacturer (BioGenes GmbH, Berlin, Germany). The cross-reactivity (CR) percentage of the antibody was calculated from 14 commercially available steroids (Sigma—Aldrich, St. Louis, MO, USA) via the following equation:

$$CR\ \% = IC50_{PdG}/IC50_{steroid} x100\%,$$

where $IC50_{PdG}$ and $IC50_{steroid}$ are the concentrations of steroids needed for a 50 % reduction in the absorbance of the maximum binding well. The method detected 100 % CR with PdG, 25 % CR with pregnanediol, 3 % CR with 17β-pregnanetriol, 3 % CR with testosterone, and less than 1 % CR with all the other steroids tested (cortisol, cortisone, dehydroepiandrosterone, E1, E2, E3, E1 glucuronide, E1 sulphate, P4, and pregnenolone). The microplates were precoated with goat anti-rabbit IgG, followed by a washing step as in the E2G ELISA. A custom PdG antibody was diluted 1:50 000 in the abovementioned sample buffer, and 100 µL of the dilution was added to

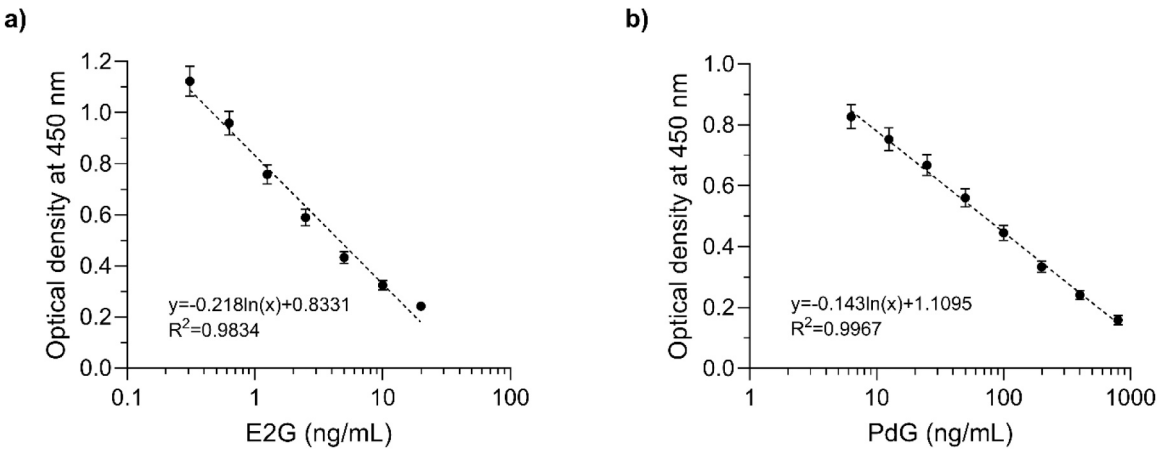

Fig. 1. The average ± standard error of the mean of standard curves used to determine concentrations in a) 17β-oestradiol-3-glucuronide (E2G) assays of 17 ELISA plates and b) pregnanediol-3-glucuronide (PdG) assays of 38 ELISA plates.

each well. The plates were sealed with adhesive tape, incubated at room temperature for an hour, and washed three times with the abovementioned washing buffer. An eight-point 1:2 standard dilution series of PdG (Sigma—Aldrich, St. Louis, MO, USA) from 800 ng/mL to 6.3 ng/mL was prepared in standard urine. The PdG standards and urine samples were diluted 1:2 in ultrapure water, and the dilutions were added at 50 μL per well in two replicates. Standard urine wells without added PdG were used to detect the maximum binding of horseradish peroxidase-labelled PdG (PdG-HRP; FKA-333, Cosmo Bio Co., Ltd., Tokyo, Japan) in the assay. PdG-HRP was diluted 1:500 in sample buffer, and 50 μL was added to each well. The sealed plates were incubated at room temperature for one hour and washed as above, followed by the addition of the substrate solution and measurement as in the E2G ELISA.

2.5. Analytical performance

A steroid hormone metabolite dilution series was used as a standard curve in each 96-well plate. All plates included a dilution series in two replicates, and the average of the replicates was used to determine the standard curve on a logarithmic scale. The averages with standard errors of the means (SEMs) of all standard curves in the E2G and PdG assays are presented in Fig. 1.

The limits of detection for the assays determined at 90 % binding (inhibition concentration, IC90) were 2.0 pg/well (20.4 pg/mL) for the E2G ELISA and 0.1 ng/well (0.97 ng/mL) for the PdG ELISA. A measuring range (IC20–80) of 0.04–4.69 ng/mL was determined for the E2G ELISA, and 2.0–145.3 ng/mL was determined for the PdG ELISA. The IC50 values were 0.46 ng/mL and 1.7 ng/mL for the E2G and PdG assays, respectively. The intra-assay and interassay coefficients of variability (CVs) were 4.2 % and 23.7 % ($n = 17$) for the E2G ELISA and 4.1 % and 24.3 % ($n = 38$) for the PdG ELISA, respectively. The variance was calculated from the absorbances of duplicate standard urine samples included in each assay.

2.6. Creatinine assay

Urinary creatinine concentrations were measured via a colorimetric method based on Jaffe's reaction. Creatinine (Merck, Darmstadt, Germany) was dissolved in ultrapure water at a concentration of 2 mg/mL on the day of the assay. The standard dilution series was prepared in water by diluting the creatinine stock solution six times at a 1:2 ratio. Ultrapure water was used as a blank control. The urine samples were diluted 1:10 in ultrapure water. Standards, blank controls, and diluted urine samples were added in two replicates of 10 μL per well in 96-well plates (655101; Greiner Bio-one GmbH, Frickenhausen, Germany), followed by 100 μL of picric acid solution (Reagena Oy, Toivala, Finland), which included ten parts of 4.5 mM picric acid and one part of 1.4 M sodium hydroxide (Reagena Oy, Toivala, Finland). The absorbances at 490 nm were measured 5 min after adding picric acid solution. Urinary creatinine concentrations were calculated via linear regression analysis ($y = kx + b$) and multiplied by the dilution factor.

To avoid errors due to variations in sample collection, samples with a creatinine concentration of less than 0.3 mg/mL (2.0 %) were excluded. In the included data set, the urinary creatinine concentration varied between 0.3 mg/mL and 4.9 mg/mL (average 1.8 mg/mL). For each sample, the urinary E2G and PdG-to-creatinine ratios were determined, and all hormone metabolite concentrations were expressed as ng/mg of creatinine (ng/Crmg).

2.7. Statistics

As the E2G and PdG data consisted of repeated measurement series of multiple female dogs, a dependency structure between the measurements had to be considered when modelling the phenomenon. To account for these dependencies and potential heteroscedasticity in the data, a linear mixed model with the necessary variance and correlation structures was used for the analysis. In the systemic part of the model, the relationship between time and response using natural splines was established (Harrell, 2015). The number and location of the knots for the splines were chosen according to recommendations (Harrell, 2015). The model for dog i, measurement series j within the dog, and measurement k was formulated as:

$$y_{ijk} = \mathbf{x}'_{ijk}\beta + a_i + b_{ij} + \epsilon_{ijk},$$

where $\boldsymbol{x}'_{ijk}\boldsymbol{\beta}$ represents the systematic part of the model, consisting of the effect of time formulated via natural splines. The random effect for dog i is denoted by a_i, and b_{ij} is the random effect for measurement series j within dog i. To model the heteroscedasticity of the residuals ε_{ijk}, we used a power-type function $|\cdot|^{2\theta}$, where θ is the variance function coefficient. The correlation between the residuals within the measurement series was modelled using an appropriate ARMA process. We used standard diagnostic plots to verify that our model adequately meets the assumptions of a linear mixed model.

A total of 196 E2G results of nine female dogs and 591 PdG results of 50 female dogs were included in the models. The fitted models were used to create predictions, i.e., estimates of expected values and 95 % confidence intervals (CIs) for the estimated values of the responses of Days 0–27. The model was fitted using REML with the nlme package (Pinheiro et al., 2022) of R software version 4.2.2 (R Core Team, 2022). For the model diagnostic, the package lmfor version 1.6 (Mehtätalo and Kansanen, 2022) was used. The descriptive values of the female dogs are expressed as averages ± SDs.

3. Results

Urine sample collections started earliest at the onset of sanguineous vaginal discharge, which was considered as Day 0, and only samples collected during the next four-week period were included in the study. A total of 603 samples were collected from the first

urination of the day in 43 female dogs in their 50 reproductive cycles. Sample collector noted and removed grass or other debris from three samples. Additional 54 samples were excluded from the study due to not being collected from the first urination of the day, along with one sample that was retrieved from the floor. Twelve samples (2 %) were excluded due to a low creatinine concentration, resulting a total of 591 urine samples for the study. Detailed information of the samples included are listed in Supplementary Table S.1.

Fitted linear mixed models were used to estimate the urinary E2G and PdG concentrations of female dogs in their natural reproductive cycles. According to the model, the expected E2G concentration was estimated to peak 6 days after the onset of sanguineous vaginal discharge. The E2G concentration increased from Days 0–6 to its maximum concentration (69 ± 18 ng/Crmg (±CI)) and decreased to approximately one-third of the maximum concentration within a week (Fig. 2).

The estimates of the expected PdG concentrations were low (21 ± 10 ng/Crmg (±CI)) at the onset of sanguineous vaginal discharge and increased slowly thereafter (Fig. 2). The urinary PdG concentration doubled on Day 8 (44 ± 10 ng/Crmg (±CI)), reached its maximum concentration (89 ± 19 ng/Crmg (±CI)) at Days 19–20 and began to slightly decrease thereafter. It needs to be noted that when interpreting the estimates of the metabolite concentrations, the limited number of results after three weeks resulted in a very wide CI.

For evaluating the suitability of measurement of concentrations of E2G and PdG for monitoring the ovarian function in female dogs, the estimates of the expected concentrations of E2G and PdG were created for Days 0, 8 and 18 and for Days 0, 9 and 18, respectively. According to the literature (Concannon, 2011), the onset of sanguineous vaginal discharge (= Day 0) was considered as the average onset of pro-oestrus, Day 8 was considered as the peak E2G concentration, Day 9 was considered as the average onset of oestrus of female dogs, and Day 18 as the onset of dioestrus. The estimates of the expected concentrations and 95 % CIs are given in Table 1.

Female dogs expressed standing oestrus in 44 of the 50 reproductive cycles. After the onset of sanguineous vaginal discharge, the onset of standing oestrus was observed on average on the tenth day (average Day 10 ± 4, range Day 5–Day 15), and which lasted approximately for 4 days (average 3.9 ± 2.3 days, range 1–8 days, $n = 29$). In fifteen cases, the last urine sample was collected during standing oestrus, so it is not known whether standing oestrus continued after this and thereby these cases were excluded from the calculation of the length of the standing oestrus.

During the 50 reproductive cycles, the female dogs were mated ($n = 23$; 46 %), inseminated ($n = 6$; 12 %), or neither mated nor inseminated ($n = 21$; 42 %). Matings were initiated on average on Day 11 (average Day 11 ± 2, range Day 6–Day 16), and inseminations were initiated on average on Day 15 (average Day 15 ± 1, range Day 13–Day 17) after the onset of sanguineous vaginal discharge. On the day of the first mating, the average urinary PdG concentration was 62 ± 39 ng/Crmg (±SD) (range 10–156 ng/Crmg, $n = 20$; three missing samples). On the day of insemination, the average concentration of urinary PdG was 119 ± 63 ng/Crmg (±SD) (range 75–208 ng/Crmg, $n = 3$; three missing samples). To our knowledge, 21 female dogs became pregnant (17 mated and four inseminated females; pregnancy rate of 72.4 %), and 116 puppies were born, confirming the fertility of the reproductive cycles.

4. Discussion

To our knowledge, the present study describes for the first time the development of ELISA methods for measuring the concentrations of E2G and PdG in female dog urine. The estimations of urinary E2G and PdG profiles resembled the serum/plasma oestrogen and P4 profiles described in the literature (Concannon, 2011). According to the fitted model of E2G, the E2G concentration peaked on Day 6 and decreased significantly from the Day 8 to Day 18 (Day 0 = the onset of the sanguineous vaginal discharge), indicating decreasing urinary E2G concentrations towards the end of the follicular phase. As the 95 % CIs of the E2G concentrations overlapped between the Days 0 and 8, the model could not detect significant increase in the E2G during that time. The expected PdG concentrations were low during the follicular phase and increased thereafter, which is consistent with the serum/plasma P4 concentrations

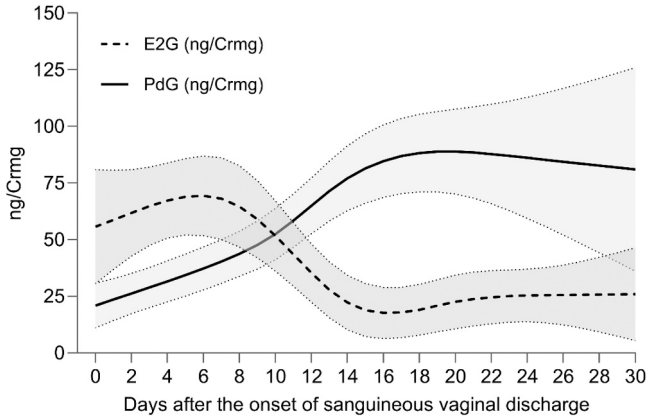

Fig. 2. Fitted linear mixed models of urinary 17β-oestradiol-3-glucuronide (E2G) and pregnanediol-3-glucuronide (PdG) concentrations of 9 (196 samples) and 43 (591 samples) female dogs, respectively. Models are aligned according to the onset of the sanguineous vaginal discharge (= Day 0). The dashed line represents the fitted model for E2G (ng/Crmg) and the solid line for PdG (ng/Crmg). The grey areas represent concentrations between the 95 % confidence interval.

Table 1
Estimates of expected values of 17β-oestradiol-3-glucuronide (E2G) and pregnanediol-3-glucuronide (PdG) concentrations (ng/Crmg) with 95 % CIs on Days 0 (considered as the average onset of pro-oestrus), 8 (considered as the average occurrence of the E2G peak) or 9 (considered as the average onset of oestrus), and 18 (considered as the average onset of dioestrus).

Day	Expected E2G±CI (ng/Crmg)	Expected PdG±CI (ng/Crmg)
0	56 ± 25	21 ± 10
8	65 ± 18	
9		48 ± 11
18	19 ± 11	88 ± 17

described in the literature (Concannon, 2011; Hollinshead and Hanlon, 2019).

Although the concentration of urinary PdG increased during the observation period, considerable differences were detected in the concentrations of PdG between the individual female dogs. According to Haase et al. (2016), plasma cortisol concentrations scale predictably with body mass in mammals, with smaller species exhibiting proportionally higher levels due to elevated mass-specific metabolic rates. This scaling relationship implies a faster turnover and clearance of steroid hormones in smaller mammals, consistent with broader metabolic principles. As cortisol is a steroid hormone, similar scaling principles might be expected for other steroids such as progesterone. In our study, however, notable individual variation in urinary PdG concentrations was observed even within breeds of similar size (e.g., six Labrador Retrievers, three Spanish Water Dogs, and three Shetland Sheepdogs). This suggests that body size alone may not be a dominant factor influencing progesterone metabolism or clearance. Given the limited number of individuals per breed, we refrain from drawing firm conclusions regarding the underlying causes of this variability. Further investigations are warranted to elucidate these differences. In addition, in relation to the timing of the E2G excretion peak, concentration of PdG seemed to increase rather early and reached its maximum concentration rather late. The phenomenon was detected occasionally at individual level also, but the reason for it remained unclear. These results indicate that the PdG assay in its current state of development would benefit of improvements in its ability to accurately discriminate the initiation of the luteal activity in female dogs.

The duration and expression of signs of pro-oestrus and oestrus vary considerably between individual female dogs (Bouchard et al., 1991). According to Hori et al. (2012), ovulation may occur already three or as late as 31 days after the onset of the sanguineous vaginal discharge, rendering the date of the onset of sanguineous vaginal discharge a weaker indicator for Day 0 compared to LH peak in aligning the concentration results. In our field study, sanguineous vaginal discharge was the only method for aligning all the concentration results without prior knowledge of the hormonal status of the female dogs. Vaginal discharge was observed throughout all reproductive cycles; however, the average duration of the discharge could not be reliably calculated. The duration of vaginal discharge varied markedly among individuals, with the shortest recorded period being 5 days and the longest extending to 29 days. These findings are consistent with previous reports of substantial variability in the expression of signs of pro-oestrus and oestrus in female dogs (Bouchard et al., 1991). In 27 out of 50 cycles, however, discharge was still visible at the time of the final sample collection, introducing uncertainty regarding the actual length of bleeding beyond the sampling period in our research. Although the investment of the volunteer dog owners in our study was remarkable, it must be considered that there could have been differences in the owners' abilities to detect and recognise signs of oestrus in their female dogs. In four of the 50 reproductive cycles (8 %), visible clinical signs of oestrus such as standing, displaying the vulva and turning the tail neither occurred nor the dog owners recognised the signs. Standing oestrus was therefore not applicable for time alignment, as it could not be detected in all female dogs, and teasing by males is not considered a reliable indicator of the timing of ovulation (Bouchard et al., 1991). Also, vaginal secretions can pass unnoticed by the owners for several reasons: the discharge can be very minor or otherwise unnoticeable, for example, due to diligent licking. Importantly, although the ability in detection of signs of oestrus varied among participants, the primary aim of this study was to evaluate the potential of urinary E2G and PdG measurements for monitoring of ovarian function in female dogs. While preliminary conclusions can be drawn despite some uncertainty or inconsistency in identifying behavioural indicators of oestrus, concurrent analysis of urinary and serum/plasma samples is necessary to confirm progesterone metabolism and excretion into urine.

In the performance evaluations, the lowest concentrations detected by the E2G and PdG ELISA methods were 2.0 pg/well and 97 pg/well, respectively. The repeatability of both ELISA methods was good (intra-assay CV of 4 % for both methods), but the reproducibility was rather limited (interassay CVs of 24 % and 29 % for E2G and PdG, respectively). In the literature, both the intra-assay and interassay CVs of PdG ELISAs have generally been reported to be $\leq$ 17 % (O'Connor et al., 2003; Hama et al., 2009; Binnie et al., 2017; Muren et al., 2017; Gifford et al., 2018). In one published study on E2G ELISA, inter- or intra-assay CVs were not reported (Muren et al., 2017), but the corresponding CVs of urinary E1–3-glucuronide ELISAs were less than 16 % (Cooke et al., 2007; Hama et al., 2008). For monitoring of ovarian function, it would be necessary to validate and improve the reproducibility of the assays. Besides to reproducibility issues, the development of the PdG ELISA encountered limitations. Owing to sensitivity problems, only a 1:4 dilution was used for the urine samples. A large amount of urine in a well leads to an increased number of interfering molecules and affects the sample matrix, which is a feasible cause of variation in the PdG assay. To minimise the possibility of errors due to the sample matrix effect of the PdG assay, a standard curve was prepared in diluted standard urine. Although the stability of the steroid metabolites was not investigated, a notable decrease in metabolite concentrations over time was not detected during the study. According to the literature, urinary E1 glucuronide, E1 sulphate and PdG are known to be relatively stable for at least two years in samples stored frozen and after up to ten freeze–thaw cycles, and even at room temperature for short time periods (O'Connor et al., 2003). No published stability data were found for urinary E2G, but in a study of Indapurkar et al. (2019), three *in vitro* synthetised E2 metabolites

(E2G, E2–17-glucuronide, and E2–3-sulphate) were found to remain stable for at least three freeze–thaw cycles. As steroid metabolites seem to be rather stable, it was assumed that non-standardised freezing conditions during sample collection or varying storage periods were not a major limitation in the study.

5. Conclusion

Albeit minor limitations, our results were promising for non-invasive monitoring of ovarian function in female dogs. Especially the further improved urinary PdG assay will be a noteworthy method for investigation of the luteal activity. Correspondence of the E2G and PdG models to the existing literature was encouraging, and particularly E2G method seemed to behave well in its current state. More studies, however, are needed to verify whether urinary PdG and circulatory P4 correspond with each other to the extent that urinary PdG measurements can be used for non-invasive monitoring of oestrus.

CRediT authorship contribution statement

Tia Pennanen: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Heli Lindeberg:** Writing – review & editing, Writing – original draft, Conceptualization. **Mika Hujo:** Writing – original draft, Methodology, Formal analysis. **Sari Viitala:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Jaakko Mononen:** Writing – original draft, Conceptualization. **Jouko Vepsäläinen:** Writing – original draft, Conceptualization.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tia Pennanen reports financial support was provided by Vetcare Oy. Sari Viitala reports financial support was provided by Vetcare Oy. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2025.107973.

Data availability

The data is available from the corresponding author on reasonable request.

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