The use of serum progesterone and prostaglandin $F_{2\alpha}$ metabolite levels to predict onset of parturition in the bitch

J.O. Nöthling[a], C.J. Joonè[b] and K.G.M. De Cramer[a]*

[a]Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110, South Africa

[b]College of Public Health, Medical and Veterinary Sciences, Division of Tropical Health and Medicine, James Cook University, Townsville, Queensland, 4817, Australia

*Corresponding author. Email address: kdcramer@mweb.co.za. Postal address: P.O. Box 704, Rant-En-Dal, Mogale City, Gauteng, 1751, South Africa.

Email addresses: kdcramer@mweb.co.za (K.G.M. De Cramer), jonothling@gmail.com (J.O. Nöthling), carolynne.joone@jcu.edu.au (C.J. Joonè)

Abstract

The prediction of time to onset of parturition in a preparturient bitch is of great clinical value, particularly for bitches at high risk of dystocia and those lacking relevant clinical data from the time of breeding. In a previous study, four cut-offs for plasma progesterone levels, measured by radioimmunoassay, were shown to be useful for predicting the likelihood of a bitch entering stage one of parturition within defined time intervals. The first aim of the current study was to evaluate these cut-offs in a clinical setting, using serum progesterone samples drawn from preparturient bitches 12-hourly instead of 6-hourly and assessed using chemiluminescence immunoassay. Furthermore, the use of 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$, (PGFM), a metabolite of prostaglandin $F_{2\alpha}$, in predicting the time to onset of parturition was evaluated. Forty bitches carrying two or more foetuses were admitted to a specialist veterinary reproduction hospital 53 d after the onset of cytological dioestrus when

that date was known, or 57 d after the last mating. Vaginal speculum examinations were performed every 6 h until cervical dilatation was visualised (time of cervical dilatation; TCD). Serum samples were collected at 08h00 and 18h00 daily until TCD. All bitches underwent elective caesarean section at TCD. Results of this study show that approximately 5% and 10% of preparturient bitches will reach TCD within 12 h despite a serum progesterone level of at least 15.8 nmol/L and 8.7 nmol/L respectively. In addition, there is a 95% probability that a preparturient bitch will reach TCD within 48 h if her serum progesterone level is below 8.7 nmol/L, and a 91% probability of her reaching TCD within 24 h if her serum progesterone level is below 3.18 nmol/L. Around 90% of bitches that demonstrate a 20% increase in PGFM over a 12-hour period are likely to be within 36 h of TCD. These results provide useful benchmarks for the management of canine parturition.

Key words

Dog, Whelp, Vaginoscopy, Luteolysis, Chemiluminescence immunoassay, PGFM

1. INTRODUCTION

Knowing when a pregnant bitch is likely, or unlikely, to whelp is of great practical importance. For breeders, this informs and potentially limits periods when intense observation of the bitch may be required. For veterinarians, knowing when a gestation will reach full term assists clinicians wishing to avoid instituting obstetrical interventions too early or too late, both of which carry significant risk for the litter.

In the bitch, estimating the day of whelping from mating dates is unreliable, given that gestation length from a single mating varies from 58 to 72 days (Concannon et al., 1983). Clinical information obtained during oestrous monitoring and breeding of a bitch can be used to estimate the date of parturition with relative precision (De Cramer & Nöthling, 2017).

However, clinicians are often presented with a late term bitch for which such information is not available or incomplete.

Several modalities for predicting the time to onset of parturition in the bitch have been explored. Radiology of late term foetuses provides an estimate of developmental stage but lacks precision in estimating time to parturition (reviewed by Lopate (2008)). Similarly, ultrasonographic morphometry of the developing foetus is considered to be of decreasing usefulness for predicting the date of whelping as gestation progresses into the third trimester (Kutzler et al., 2003). Recently, the lung-to-liver ratio of mean grey levels of foetal lung and liver showed potential in identifying the last week of gestation (Banzato et al., 2017). In addition, ultrasonographic evidence of foetal gastrointestinal motility has been suggested to be indicative of foetal maturity, however this technique is considered unreliable as a sole predictor of impending parturition (Gil et al., 2015; Milani et al., 2020).

Early research suggested that a transient drop in the rectal temperature of a late pregnant bitch is indicative of impending parturition (Concannon et al., 1977; Tsutsui et al., 1982). However, more recent research found that body temperature measured vaginally or rectally was not clinically useful for predicting imminent parturition (Geiser et al., 2014; Veronesi et al., 2002).

It has been well established that circulating levels of progesterone (P4) decline prior to parturition in the bitch (Brugger et al., 2011; Concannon et al., 1978; De Cramer et al., 2018; England et al., 1996; Veronesi et al., 2002). Recently, De Cramer et al. (2018) identified four cut-offs for plasma P4 levels that are helpful in predicting the time to onset of parturition in the bitch. The authors determined plasma P4 levels, using radioimmunoassay (RIA), 6-hourly prior to the time of uterine cervical dilatation (TCD) in 25 bitches. They concluded that bitches that were within 12 h of TCD have a 2% probability (95% CI 0% to 10%) of having plasma P4 levels of 15.8 nmol/L or above and a 6% probability (95% CI 2% to 16%) of

having levels of 8.7 nmol/L or above. They also concluded that bitches with plasma P4 levels below 8.7 nmol/L have a 99% probability (95% CI 94% to 100%) of reaching TCD within 48 h and those with levels below 3.18 nmol/L have a 100% probability (95% CI 90% to 100%) of reaching TCD within 24 h.

Unlike other domestic species, dioestrus in the non-pregnant bitch is relatively prolonged, with regression of corpora lutea appearing to be a function of age rather than active luteolysis. In contrast, regression of corpora lutea in the late pregnant bitch is accelerated as the bitch approaches parturition by luteolysis induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha;}$ Kowalewski (2014)). During the period of prepartum luteolysis, low circulating levels of P4 coincide with high circulating levels of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM), a major metabolite of $PGF_{2\alpha}$ (Kowalewski et al., 2010). Circulating PGFM levels in the bitch start to rise 1–2 days prior to parturition, continue to rise and reach peak values during parturition and decline thereafter. During this rise, levels differ widely among bitches (Concannon et al., 1988; Veronesi et al., 2002) .

The first aim of the current study was to assess the utility of the criteria identified by De Cramer et al. (2018) for predicting the onset of parturition in bitches in a clinical setting, using serum P4 levels determined by chemiluminescence immunoassay (CLIA) and with bitches bled in the morning and early evening only. The second aim was to determine whether serum PGFM levels have potential application in clinical practice as a predictor of TCD in preparturient bitches.

2. MATERIALS AND METHODS

This research was approved by the Animal Ethics Committee of the University of Pretoria (project V106-18).

2.1 Animals and sampling

Forty bitches (30 Boerboel, five English bulldog, four American bully and one Labrador retriever) that were admitted to a specialist veterinary reproduction hospital for prepartum monitoring and elective caesarean section were studied. All bitches carried at least two foetuses. Bitches were admitted 53 d after the first day of cytological dioestrus (Holst et al., 1974) when that day was known, or 57 d after the last mating. They were housed in indoor cages and taken for a walk outdoors twice daily. Feed consisted of a commercial dry pellet. All bitches had access to clean water *ad lib*.

Cervical dilatation marks the onset of stage one of parturition and indicates that caesarean section can be performed safely (De Cramer, Joubert, et al., 2017; Smith, 2007). Therefore, each bitch underwent vaginoscopy as previously described (De Cramer et al., 2018, 2019) every 6 h, from admission until dilatation of the uterine cervix was first observed (time of cervical dilatation; TCD). If it was suspected that TCD was imminent, based on behavioural signs described previously (De Cramer et al., 2019), vaginoscopy was performed more frequently.

Blood samples were collected twice daily, within 30 minutes of 08h00 and 18h00 respectively, until TCD. At each sampling point, blood was collected into serum tubes with clot activator (BD Vacutainer®, BD Plymouth, UK) and centrifuged once clotting had occurred. Serum was transferred into uniquely labelled cryovials (Catalogue number 750273, PlastPro Scientific, Edenvale South Africa) and frozen at -20°C to -80°C until required for analysis.

The time at which each serum sample was obtained, relative to TCD, was classified as follows: Time 1 accounted for sera obtained at the last regular bleeding before TCD, Time 2 for sera obtained at the penultimate regular bleeding before TCD, and so on. For simplicity, although the intervals between regular bleeding times were, approximately, either 10 h or 14 h, and intervals between the last regular bleedings and TCD were either $\leq$ 10 h or $\leq$ 14 h, we

assumed intervals of ≤ 12 h (Table 1). Only sera collected up to 60 h prior to TCD (Time 5) were included in the study.

Each bitch underwent elective caesarean section once final blood samples had been collected at TCD. The number of puppies delivered alive or stillborn and the number of puppies alive 2 h post-delivery were recorded.

2.2 Measuring progesterone levels

Serum P4 levels were determined using CLIA (Immulite 2000; Siemens Healthcare Diagnostics Products Ltd., Glyn Rhonwy, UK). The same kit was used for all assays, which were performed in three runs over three days. Each serum sample was assayed in duplicate within a single run; ImmP4 refers to the mean of each pair of duplicates. The P4 levels of samples in duplicate pairs were used to determine the intra-assay coefficient of variation (CV). The interassay CV was determined by re-analysing 12 selected serum samples from the first run within the second and third runs.

2.3 Measuring PGFM levels

Serum PGFM levels were analysed using the DetectX 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) enzyme immunoassay kit (Arbor Assays, Michigan, USA; (Tamminen et al., 2019)). The same kit was used for all assays, which were performed in five runs over five days. Each serum sample was assayed in duplicate in a single run. The intra-assay CV was determined using the PGFM levels of each pair of replicates. The term PGFM henceforth refers to the mean PGFM level of each pair. The inter-assay CV was determined by re-analysing six serum samples (two with low levels, two with medium levels and two with high levels) from the first run in at least two further runs.

2.4 Data analysis

2.4.1 Applying the cut-offs reported by De Cramer et al. (2018) to predict TCD using ImmP4 measured 12-hourly

In order to assess the utility of the cut-offs defined by De Cramer et al. (2018), we determined the probability of bitches that had not yet reached TCD but were within 12 h thereof of having ImmP4s of 15.8 nmol/L or above, or 8.7 nmol/L or above. We also determined the probability of bitches having ImmP4s below 8.7 nmol/L being within 48 h to TCD. Similarly, we determined the probability of bitches having ImmP4s below 3.18 nmol/L being within 24 h to TCD. We compared these probabilities to the 95% compatibility intervals reported by De Cramer et al. (2018).

2.4.2 Assessing PGFM as a possible predictor of TCD

We assayed PGFM in four sequential serum samples, with the last having been collected no less than 12 h (actually 10–14 h) prior to TCD and the first no less than 48 h prior to TCD. Sequentially, we labelled these PGFM levels PGFM12, PGFM24, PGFM36 and PGFM48. We noted that, as expected, PGFM levels varied widely among bitches and therefore decided to use the relative change in PGFM levels between consecutive bleeding times in a bitch, rather than the absolute change as a basis for comparison among bitches. We determined the relative change by dividing each raw PGFM level by the one before to obtain the following quotients: Quotient1224 = PGFM12 / PGFM24, Quotient 2436 = PGFM24 / PGFM36 and Quotient 3648 = PGFM36 / PGFM48. We inspected a scatterplot of the quotients against h prior to TCD for potentially useful cut-off points.

2.4.3 Point estimates and the 95% compatibility interval

The distribution of data was described as the mean (point estimate) with its 95% compatibility interval (95% CI) (Amrhein et al., 2019). The distribution of coefficients resulting from a regression analysis was described as the expected coefficient (point estimate) with its 95% compatibility interval (95% CI). A proportion was described as the expected

proportion with its 95% compatibility interval. Values falling within the limits of a 95% compatibility interval were deemed compatible with the data (Amrhein et al., 2019).

All analyses were done with STATA version 14 (StataCorp, College Station, Texas, USA).

3. RESULTS

In 18 of the 40 bitches, D1 from the preceding heat was known. The median duration of hospitalisation prior to caesarean section in these bitches was 2.7 d (range 1.8–4.3 d). The median duration of hospitalisation prior to caesarean section in the remaining 22 bitches, for which D1 was not known, was 4.2 d (range 1–11.3 d). The 40 caesarean sections yielded 307 puppies of which eight (3%) from seven litters were stillborn and six (2%) from four litters died before 2 h (Mean litter size: 7.68, SD 3.68, n=40; mean number stillborn per litter: 0.2, SD 0.464, range 0 to 2; mean number that died before 2 h: 0.15, SD 0.483, range 0 to 2.). Additional information regarding these results according to breed is provided in Supplementary Tables 1 and 2.

3.1 Intra- and interassay coefficients of variation obtained with the Immulite progesterone assay

The intra- and interassay CVs for P4 levels measured with Immulite are shown in Table 2.

3.2 Applying the cut-offs reported by De Cramer et al. (2018) to predict TCD using ImmP4

Except for bitches 6, 13 and 18, which were not bled at Time 5, ImmP4 was determined at each of the five regular blood sampling points prior to TCD as well as at TCD (Figure 1). De Cramer et al. (2018) concluded that a plasma P4 level of 15.8 nmol/L or above is a useful indicator that bitches are highly unlikely to reach TCD within 12 h. Applying this cut-off, 5% of the 40 bitches in this study (bitches 39 and 40 on Figure 1) had ImmP4s of 15.8 nmol/L or above at the last regular bleeding prior to TCD—that is, when they were within about 12 h of

TCD. From Figure 2, it can be discerned that these two bitches were bled 14 h prior to TCD. This proportion falls well within the 95% CI of 0–10% that De Cramer et al. (2018) found. De Cramer et al. (2018) also concluded that a plasma P4 level of 8.7 nmol/L or above is a useful indicator that bitches are unlikely to reach TCD within 12 h. Applying this cut-off, 10% of the 40 bitches in the current study (bitches 37–40 on Figure 1) had ImmP4s of 8.7 nmol/L or above at the last regular bleeding prior to TCD (Figure 2). This proportion falls well within the 95% CI of 2–16% found by De Cramer et al. (2018).

De Cramer et al. (2018) concluded that a plasma P4 level of below 8.7 nmol/L is a useful indicator that bitches are likely to reach TCD within 48 h. In the current study, ImmP4 decreased below 8.7 nmol/L before TCD in 38 of the 40 bitches (in all except bitches 37 and 39). In five of the 38 (bitches 3, 7, 8, 23 and 34; Figure 1), ImmP4 was already below 8.7 nmol/L more than 48 h prior to TCD. The 38 bitches yielded 96 ImmP4s below 8.7 nmol/L of which 91 occurred 48 h or less prior to TCD (Figure 2). Therefore, if a preparturient bitch yields an ImmP4 below 8.7 nmol/L, the probability of her being within 48 h from TCD is 91/96, or 95%, which falls within the 95% CI of 94–100% found by De Cramer et al. (2018).

De Cramer et al. (2018) also concluded that a plasma P4 level below 3.18 nmol/L is a useful indicator that bitches are likely to reach TCD within 24 h. In the current study, ImmP4 decreased below 3.18 nmol/L prior to TCD in 23 of the 40 bitches. In three of the 23 bitches (bitches 2, 3 and 9; Figure 1), ImmP4 decreased below 3.18 nmol/L more than 24 h prior to TCD. The 23 bitches yielded 32 ImmP4s below 3.18 nmol/L prior to TCD, of which three occurred more than 24 h prior to TCD (Figure 2). Therefore, if a preparturient bitch yields an ImmP4 below 3.18 nmol/L, the probability of her being within 24 h of TCD is 29/32, or 91%, which falls within the 95% CI of 90–100% that De Cramer et al. (2018) found.

3.3 Assessment of PGFM as a predictor of TCD

The intra- and interassay CVs for PGFM are shown in Table 3.

Visual appraisal of Figure 1 reveals that in most, but not all, bitches there is an obvious inverse relationship between PGFM and ImmP4, with PGFM levels rising and ImmP4 declining as TCD approaches.

Levels of PGFM measured within this study varied widely (mean 9006 pg/mL, SD 8290 pg/mL, ranging from 1019 to 52699 pg/mL). Levels of PGFM also varied widely within individual bitches (within-bitch SD ranging from 201 to 17108 pg/mL).

Given the limited use of absolute values of PGFM as a predictor of impending parturition, the level of PGFM relative to the level measured 12 h previously was expressed as a quotient. Figure 3 shows the values of quotients at various times before TCD and indicates potentially useful thresholds. If the level of PGFM increases by 20% or 30% (thresholds of 1.2 or 1.3) over a 12-hour period, which is expected to occur in approximately 85–90% (95% CI: 70–96%) of preparturient bitches, the probability that TCD will follow within 36 h is around 90% (95% CI 79–97%; Table 4).

4. DISCUSSION

The current study, which made use of a more practical schedule of measuring P4 levels twice daily, predicts the likelihood of the onset of parturition within defined intervals in agreement with the findings of De Cramer et al. (2018), who measured P4 levels four times daily. As per the findings of De Cramer et al. (2018), bitches with P4 levels of 8.7 nmol/L or above, or 15.8 nmol/L or above, are unlikely to reach TCD within 12 h. Still, about one in 10 bitches with an ImmP4 of 8.7 nmol/L or above and about one in 20 bitches with an ImmP4 of 15.8 nmol/L or above will reach TCD within 12 h.

In the current study, two of the 40 bitches showed an ImmP4 above 10 nmol/L at TCD. These results highlight the risk of relying solely on serum P4 levels to manage preparturient bitches.

In the current study, neonatal survival rates support the assertion that caesarean section is safe

once cervical dilatation has commenced and foetal membranes can be visualised (De Cramer, Joubert, et al., 2017; Smith, 2007). This suggests that vaginoscopy—a safe, simple and cost-effective tool—should be a routine procedure in the clinical management of preparturient bitches. Furthermore, if bitches have an ImmP4 greater than 8.7 nmol/ L and have other indications of being close to parturition, such as a known interval since the LH surge, ovulation or the onset of cytological dioestrus (De Cramer & Nöthling, 2017) or a relaxed perineum and paracervix of the vagina, their cervices should be assessed six-hourly with a vaginal speculum to timeously identify TCD.

The findings in this study agree with those of De Cramer et al. (2018) that about 95% of ImmP4s below 8.7 nmol/L in preparturient bitches will be from bitches that are within 48 h of TCD. They also agree with De Cramer et al. (2018) that about 90% of ImmP4s below 3.18 nmol/L in preparturient bitches are from bitches that are within 24 h of TCD. Yet, in this study, about one in eight bitches with ImmP4s below 8.7 nmol/L took longer than 48 h to reach TCD and the same proportion of bitches with ImmP4s below 3.18 nmol/L took longer than 24 h to do so. In the current study, one bitch (Bitch 34) showed an ImmP4 below 6 nmol/L at admittance, over 57 h prior to TCD. Her ImmP4 profile prior to this timepoint is unknown. England et al. (1996) reported one bitch that had a serum P4 level below 6.36 nmol/L (2 ng/ml) for 6 d prior to parturition and Austad et al. (1976) reported one bitch with undetectable P4 levels for 10 d prior to parturition. Although likely rare, these cases again highlight the need for caution when evaluating serum P4 levels in preparturient bitches.

The values of PGFM reported in the current study (mean 9006 pg/ml, SD 8290 pg/ml) are substantially higher than those reported by Concannon et al. (1988) (mean at peak 3640 pg/ml, SD 1996 pg/ml) but comparable to those reported by Veronesi et al. (2002) (mean 24 h prior to parturition 11418 pg/ml, SD 5035 pg/ml). Concannon et al. (1988) and Veronesi et al. (2002) showed that serum PGFM levels only start rising from around 48 h prior to

parturition. In order to assess an early rise in PGFM as a timely predictor of TCD, we started determining PGFM levels between 48 and 60 h prior to parturition. Concannon et al. (1988) and Veronesi et al. (2002) also showed that PGFM levels continued to rise until or beyond the onset of parturition. Because our aim was to predict TCD using 12-hourly assessments of PGFM levels and keeping in mind the practical constraints of sample transit and testing through a laboratory, we did not measure PGFM levels at TCD or less than about 12 h prior to TCD.

This study, which is the first to report on the use of PGFM levels as a predictor of the time to parturition, shows that, while absolute PGFM levels are not clinically useful, relative PGFM levels do have clinical utility. Between 60 and 12 h prior to TCD, PGFM levels varied widely within and among bitches. Interference by serum indices (haemolysis, icterus and lipaemia) (Hasanato et al., 2015), or some other sample constituent, may have contributed to this variation. Nevertheless, the absolute levels of PGFM are currently of no value for predicting the time to parturition. However, comparing a PGFM level to the level 12 h previously is useful. An increase in PGFM levels by as little as 10% during a 12-hour period is expected to occur in 93% of bitches within 60 h of TCD and an expected 84% of such increases would occur during the 12-hour period ending 24 or 12 h prior to TCD and not earlier. An increase of more than 20% in PGFM levels during a 12-hour period is an optimal threshold as an expected 90% of bitches within 60 h of TCD would have at least one rise above the threshold, while an expected 90% of rises above 20% would occur over the 12-hour period ending 24 h or 12 h prior to TCD and not earlier. In light of the above, any rise in PGFM level by more than 10%, preferably 20%, over a 12-hour period provides a strong indication that parturition, as indicated by the onset of dilatation of the uterine cervix, should start within 36 h.

In conclusion, the current study further establishes the utility of cut-off values for circulating P4 proposed previously (De Cramer et al., 2018) in the clinical setting, with sampling

299 performed 12-hourly rather than 6-hourly and P4 analysed via CLIA rather than RIA. In
300 addition, this study found that absolute PGFM levels were of no clinical utility for predicting
301 the interval to onset of parturition. However, an increase of at least 10%, preferably 20%, in
302 circulating PGFM levels over a 12-hour period in a preparturient bitch provides strong
303 evidence that stage one of parturition will ensue within 36 hours of the final serum sample
304 being drawn.
305
306 **Figure legends**
307 **Fig. 1** Serum levels of progesterone and prostaglandin $F_{2\alpha}$ metabolite (PGFM) in each of 40
308 preparturient bitches that were bled twice daily at 08h00 and 18h00 prior to cervical
309 dilatation, as well as at cervical dilatation (TCD, shown as Time 0).
310 **Fig. 2** Serum progesterone levels determined with a chemiluminescence immunoassay
311 (Immulite 2000; ImmP4) at regular times every day (08h00 and 18h00) and at the time of
312 cervical dilatation (TCD; Time 0) in 40 bitches. The data are aligned to TCD.
313 **Fig 3.** Prostaglandin $F_{2\alpha}$ metabolite (PGFM) quotients relative to the time of cervical
314 dilatation (TCD; Time 0) in 40 bitches. The data are aligned to TCD.
315
316 **Author contributions**
317 JON: Conceptualization, Methodology, Formal analysis, Supervision, Writing – Original
318 Draft. KGM: Investigation, Resources, Supervision, Writing – Original Draft. CJJ: Writing –
319 Review & Editing
320
321 **Conflicts of interest**
322 The authors have no conflicts of interest to declare.
323

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