Absence of a corpus luteum and relatively lesser concentrations of progesterone during the period of pre-ovulatory follicle emergence results in lesser pregnancy rates in *Bos indicus* cattle

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ABSTRACT

Concentrations of progesterone before AI have had variable effects on fertility in both *Bos indicus* and *Bos taurus* cattle. The aim of this study was to determine if fertility and concentrations of progesterone after AI were affected in *Bos indicus* females when pre-ovulatory follicles develop in the absence or presence of a corpus luteum (CL). Between 6.5–7.5 days after a synchronised oestrus, all follicles ≥ 4 mm in diameter were aspirated (Day 0) and cloprostenol was administered on Days 0 and 1 (LP4, $n = 36$) or on Days 4 and 5 (HP4, $n = 40$). Animals were inseminated on detection of oestrus until Day 9. Breeding continued using natural mating between Days 9 and 19, AI on detection of oestrus between Day 19 and 29 and natural mating between Days 29–90. Mean concentrations of progesterone were less on Days 2–4 in the LP4 compared to the HP4-treated animals but similar on Days 14 and 20. In the LP4- compared to the HP4-treated animals, the odds of being detected in oestrus and ovulating close to the first AI were similar, but odds of pregnancy to first AI (OR = 0.19, 95% CI 0.07 – 0.52) and after 1, 4 and 13 weeks of breeding were less ($P ≤ 0.051$). Absence of a CL and relatively lesser concentrations of progesterone during emergence of pre-ovulatory follicles resulted in lesser pregnancy rates to AI in *Bos indicus* cattle but did not affect concentrations of P4 after ovulation.

1. Introduction

Results from several studies suggest that concentrations of progesterone before ovulation can influence fertility to AI in both *Bos indicus* (Carvalho et al., 2008; Dias et al., 2009; Claro et al., 2010; Cavalieri et al., 2018) and *Bos taurus* cattle (Bisinotto et al., 2015b; Stevenson et al., 2015; Stevenson and Lamb, 2016). Greater concentrations of progesterone before a timed AI or breeding have been associated with greater fertility in several studies (Fonseca et al., 1983; Meisterling and Dailey, 1987; Folman et al., 1990; Echternkamp and Thallman, 2011; Colazo et al., 2013; Bisinotto et al., 2015ab) and lesser rates of early embryonic loss (Wiltbank et al., 2011; Martins et al., 2018). Fertility was also less following ovulation from dominant follicles arising from the first follicular wave that emerged when there were lesser concentrations of progesterone compared to those that emerged as part of the second follicular wave when concentrations of progesterone were greater (Bisinotto et al., 2010; Denicol et al., 2012). Furthermore, with hormonal-induced super-stimulation of ovarian follicular development (Rivera et al., 2011) and following synchronisation of oestrous cycles of dairy cows (Wiltbank et al., 2011), there was a greater embryo quality when cows had ovulations from follicles that emerged when there were greater compared to lesser concentrations of progesterone. Cows with relatively lesser concentrations of progesterone during the dominance phase of follicular development also had a greater pregnancy loss (Martins et al., 2018). It has

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also been suggested that greater blood flow and steroid metabolism in the liver of high milk producing dairy cows, leading to lesser peripheral concentrations of progesterone may be one factor contributing to the lesser fertility that occurs in dairy compared to beef cattle (Stevenson and Lamb, 2016). These results indicate that if follicles emerge when there is a relatively lesser progesterone milieu the quality of embryos and fertility is generally less.

In not all studies, however, have there been consistent improvements in pregnancy rates to AI when concentrations of progesterone were greater before AI. In several studies with beef and dairy cattle with synchronised oestrous cycles, improvements in pregnancy rates to a timed AI have occurred when concentrations of progesterone were less before AI (Dias et al., 2009; Meneghetti et al., 2009; Peres et al., 2009; Martins et al., 2014) but no significant improvements were observed in other studies (Carvalho et al., 2008; Phillips et al., 2010; Dadarwal et al., 2013; Sa Filho et al., 2014; Abreu et al., 2018b). Improvements in fertility when there are relatively lesser concentrations of progesterone before AI are thought to be mediated through lesser concentrations of progesterone resulting in increased frequency of release of LH which results in an increased growth rate of pre-ovulatory follicles (Carvalho et al., 2008). This, in turn, results in an increase in the size and maturity of follicles at the time of AI (Dias et al., 2009; Peres et al., 2009), increases the proportion of animals that are in oestrus at the time of AI (Sa Filho et al., 2010; Cavalieri et al., 2018) and can increase concentrations of progesterone after AI (Vasconcelos et al., 2001; Sa Filho et al., 2010). These results indicate that potential effects of progesterone on developing follicles may be related to effects on the maturity of follicles at the time of AI, especially if timed AI occurs.

In different studies confounding factors have made it difficult to determine whether effects on fertility were attributed to changes in the maturity of ovarian follicles at the time of AI or by effects on the fertility of oocytes. These include differences in oestrous cycling status at the start of treatments and variability in whether a CL was present during treatment, differences in prevailing concentrations of progesterone during development of the pre-ovulatory follicle and during different phases of follicular development, different methods used to synchronise stage of oestrous cycles and the use of timed insemination with induction of ovulation. Thus, to further clarify the relationship between circulating concentrations of progesterone during development of the pre-ovulatory follicle and fertility there is a need to tightly and reliably synchronise pre-ovulatory follicular development in the presence of specific concentrations of progesterone.

In dairy cattle, a threshold circulating concentration of progesterone during pre-ovulatory follicular development of at least 2 ng/mL appears to be necessary for normal fertility (Bisinotto et al., 2015a; Stevenson and Lamb, 2016). In *Bos indicus* cattle, a threshold concentration for optimum fertility during development of the pre-ovulatory follicle has not been established. In *Bos taurus* beef cattle, there has been consideration that differences in, genotype and nutritional management may mean that concentrations of progesterone during development of the pre-ovulatory follicle may be less important than in dairy cattle (Stevenson and Lamb, 2016). This hypothesis is supported by recent findings in *Bos taurus* beef cattle where, as a result of supplementing with different doses of progesterone, pregnancy rates to AI were similar when there was emergence of pre-ovulatory follicles in lesser compared to greater concentrations of progesterone (Abreu et al., 2018b). Clarification of the functions of progesterone during the period of emergence of pre-ovulatory follicles on fertility in *Bos indicus* cattle is, however, still needed. The aim of this study was to determine if fertility and concentrations of progesterone after AI were affected in *Bos indicus* females in which pre-ovulatory follicles developed in the absence (low progesterone) or presence of a CL (high progesterone) when there was insemination as a result of a spontaneous oestrous expression. The results of the study should help to clarify the importance of progesterone during the period of development of the pre-ovulatory follicle in spontaneously ovulating *Bos indicus* cattle.

2. Materials and methods

2.1. Animals and treatments

This study was conducted at the James Cook University Tropical Veterinary Research Station "Fletcherview", (latitude 19°53′4″S; longitude 146°10′43″E) which is located in a seasonally dry tropical region of north Queensland. Cattle grazed native pastures such as Astrebia *spp.* (Mitchell grass), *Chrysopogon fallax* (Ribbon grass), *Bothriochloa ewartiana* (desert blue grass) and improved pastures consisting predominantly of *Cenchrus cillaris* (Buffel grass). Experimental procedures conducted in association with this study received approval from the James Cook University Experimentation Ethics Review Committee (approval number: A1479).

Bos indicus (Brahman) heifers ($n = 71$) aged 2 years of age and non-lactating cows ($n = 14$) aged 3 to 4 years of age, were allocated to an initial treatment to synchronise oestrous cycles. A diagrammatic illustration of the treatment protocol is illustrated in Fig. 1. Animals were weighed and body condition scored on a scale from 1 to 9, where 1 = emaciated and 9 = obese (Wagner et al., 1988) and treated with an intravaginal progesterone releasing insert (CIDR) for 7 days (Days -16 to -9). Oestradiol benzoate was administered at insertion (0.75 mg IM) and 24 h after removal (0.75 mg IM) of inserts while cloprostenol (0.5 mg IM) was administered at the time of removal of inserts (Day -9). Using this treatment protocol, induction of oestrus was expected 48 to 72 h after removal of inserts (Cavalieri et al., 2004).

Animals were subjected to transvaginal follicular aspiration 6.5–7.5 days after being induced into oestrus. The day of aspiration was designated as Day 0 of the study. Epidural anaesthesia was applied by administering 3–5 mL of lignocaine (Ileum Lignocaine 20 injection, Troy Laboratories, Glendenning NSW) into the sacrococcygeal space. All follicles > 4 mm in diameter were aspirated following visualisation of ovaries with a 7.5 MHZ curvilinear probe (Mylab 5; Medical Plus Australia Pty Ltd, Tullamarine, Vic) inserted into the vagina and follicular puncture with a 19 g x 3 inch disposable needle. Animals in which a CL could be visualised using transvaginal ultrasonography at the time of aspiration ($n = 82$) were stratified within age groups and then randomly allocated to treatment with cloprostenol (0.5 mg IM), either immediately following aspiration and again the following day (low progesterone
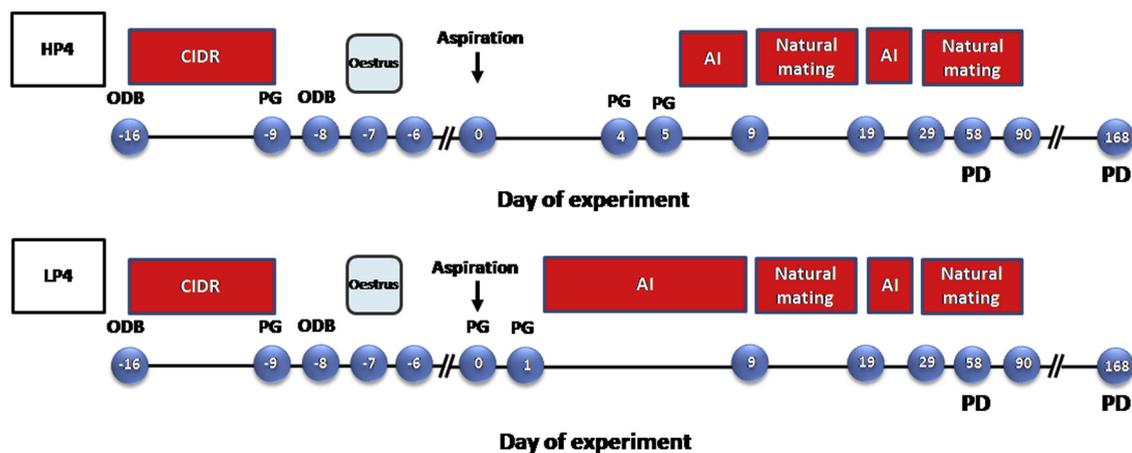
Fig. 1. Diagrammatic illustration of the treatment protocol. *Bos indicus* females first had oestrous cycles synchronised by administration of an intravaginal progesterone releasing insert (CIDR) for 7 days and administration of oestradiol benzoate (ODB) commencing on Day -16. Cloprostenol (PG) was administered on Day -9 and ODB administered 24 h later to induce oestrus and ovulation. On Day 0, females with a CL (Days 6.5–7.5 of the oestrous cycle) had all follicles ≥ 4 mm in diameter aspirated. Females were then either administered a dose of PG on Days 0 and 1 (low progesterone group, LP4) or on Days 4 and 5 (high progesterone group, HP4). Animals were then inseminated on detection of oestrus for up to 9 days. Bulls were released between Days 9–19 and 29–90. Between Days 19 and 29 animals that were detected in oestrus were inseminated as part of the commercial breeding plan employed within the herd to increase the number of AI-derived calves and not for any specific reason related to the study. Pregnancy diagnosis was conducted on Day 58, and again on Day 168.

group, LP4, *n* = 40; 34 heifers and six cows) or 4 days following aspiration and again the following day (high progesterone group, HP4; *n* = 42; 34 heifers and eight cows) to induce luteolysis.

2.2. Detection of oestrus and artificial insemination

Aids for the detection of oestrus (Estrotect, Genetics Australia, Bacchus Marsh, VIC) were applied perpendicular to the spine in the region of the sacrococcygeal joint at the time of aspiration. Animals were monitored for the onset of oestrus twice daily (07:00 to 08:00 and 17:00-18:00) from Day 1 to 9. Animals detected in oestrus were inseminated once daily each morning following the detection of oestrus (> 25% background colour of oestrous detection aid visible) from Days 0 to 8 with no animals being detected in oestrus on Day 9. Bulls (*n* = 4) that had passed a breeding soundness examination prior to the breeding season (Fordyce et al., 2006) were released with the animals from Day 9 to 19 and 29 to 90. Aids for the detection of oestrus were re-applied to all animals on Day 19. Release of bulls after the first AI occurred at least 24 h after the last animal was detected in oestrus and inseminated. Between Days 19 and 29 following follicular aspiration, animals were observed for signs of oestrus once daily and those detected in oestrus were inseminated in accordance with routine breeding management of the herd. All artificial inseminations were conducted by the same person using frozen-thawed semen from a single bull. Pregnancy diagnosis using transrectal ultrasonography (MyLab 5, 7.5 MHz probe) was conducted on Day 58, and again on Day 168. Using this pregnancy testing regimen, pregnancy stage was estimated and time of conception was classified as having occurred within 1, 4 and 13 weeks of the commencement of AI (Kastelic et al., 1988; DesCoteaux et al., 2009). Pregnancy rate to first AI was calculated by dividing the number of animals that were diagnosed as pregnant to the first AI by the number of animals that received a first AI. Pregnancy rates at 1, 4 and 13 weeks after commencement of breeding were calculated by dividing the cumulative number of animals classified as becoming pregnant within each respective time period by the total number of animals that were treated.

2.3. Ultrasonography

A subset of animals from each treatment (LP4: ten heifers and one cow; HP4: eight heifers and one cow) were randomly selected to monitor ovarian follicular development following follicular aspiration with ultrasonography. Animals within this subset within the LP4- and HP4-treatment groups were stratified by age (2.2 ± 0.2 compared with 2.2 ± 2, years, respectively, *P* = 0.888) and body weight (372.5 ± 19.8 compared with 371.2 ± 22.1 kg, respectively, *P* = 0.967) and subjected to ultrasonography of ovaries on Days 2, 4, 6–9, 14 and 20 after follicular aspiration. For every animal that was inseminated, ovaries were also examined with ultrasonography at the time of AI (MyLab 5, 7.5 MHz probe). Video recordings of each ultrasonic examination were made. All follicles ≥ 3 mm in diameter and corpora lutea were measured using electronic callipers and ovarian maps were drawn to record the diameter and number of follicles and corpora lutea present in each ovary. The length and width of follicles were recorded and the average of the two measurements was used for analyses. In the subset of animals monitored repeatedly with ultrasonography, ovulation was considered to have occurred following the sudden disappearance of follicles that were observed to emerge following aspiration with the subsequent development of a CL in the same ovary. The animals in the subgroups were maintained with the rest of the animals during the study.

2.4. Blood sampling and hormone assays

Blood samples were collected into evacuated tubes containing lithium heparin (Vacutainer®, BD North Ryde, NSW) from the coccygeal vein or artery from the 20 animals subjected to serial ultrasonography on Days 0, 2, 4, 6, 7, 14 and 20 and from all of the animals on Days 4 and 14. Following collection, blood samples were placed on ice, subsequently centrifuged and plasma separated and stored at -20 °C until the time of assay. Concentrations of progesterone in plasma were determined using a commercially available RIA kit (IBL P4 RIA, Abacus ALS, East Brisbane, Qld). The minimum detectable limit of the assay was 0.10 ng/mL and the intra-and inter-assay coefficients of variations for plasma pools of 1.1, 5.0 and 8.3 ng/mL were 7.5% and 10.6%; 7.1% and 12.0%; and 5.0% and 6.4%, respectively. The ratios for observed to expected values for dilution parallelism with the standard curve for the assay was assessed by diluting 5 samples by 75%, 50%, 25% and 12.5%. The mean $\pm$ SEM observed/expected ratio was 116.3 $\pm$ 7.9%. A minimum concentration of progesterone > 1.5 ng mL on Day 14 was used to indicate that ovulation had occurred by that day (Rhodes et al., 1995).

2.5. Statistical analyses

Statistical analyses were performed using the statistical software IBM SPSS Statistics (version 20.0). Data were reported as mean $\pm$ SEM. An analysis of variance (ANOVA) was used to compare the mean intervals to oestrus and diameters of ovarian follicles at the time of AI. Weight was initially included as a covariate but excluded from the final model if the effect was not significant. Each dependent variable was tested for normality using a Shapiro-Wilks test and examination of residual plots while homogeneity of variance was tested using Levine's test. Concentrations of progesterone on Days 4 and 14 in all of the animals were compared using a Mann-Whitney U test as variance was not homogenous across groups. In the animals that were subjected to serial ultrasonography, repeated measures analysis of variance was used to assess the effects of treatment on plasma concentrations of progesterone between Days 0 and 20, and the diameter of emerging pre-ovulatory follicles between Days 2 and 6. Models included the main effects of treatment, time and the interaction of treatment and time. Weight was included as a covariate initially but was subsequently excluded from final models as its effect was not significant. When a treatment by time interaction was detected, differences between means on individual days were compared with a t-test. Logistic regression was used to model the effect of treatment, age (heifer or cow), whether animals were part of the serially ultrasonically assessed group or not and weight on the outcomes for oestrous detection rate at first AI, pregnancy rate to AI and after 1, 4 and 13 weeks of breeding. Terms were considered for elimination from each model using backwards step-wise logistic regression, although treatment group, was considered to be fixed effect and always left in every model. The test for elimination was a likelihood-ratio test using a significance level of $P \geq 0.10$. If an interaction was significant at $P < 0.10$, the associated main effects were included in the model. Goodness of fit of the models was assessed using the Hosmer and Lemeshow test. Probability values for all main effects remaining in models were determined using the approximate chi–squared distribution of the likelihood ratio statistic. Odds ratios and 95% confidence intervals were calculated for all main effects remaining in models. Probability values < 0.05 were considered significant and $0.05 < P < 0.10$ were regarded as a tendency.

3. Results

3.1. Exclusions

Three animals were removed from the study on the day of folliclular aspiration as no CL were visible in the ovaries, leaving 82 animals subjected to follicular aspiration. After follicular aspiration there were follicles of five animals where functionality of these follicles appeared to be sustained, resulting in animals being detected in oestrus within 3 (LP4, $n = 3$) and 4 (HP4, $n = 1$) days of aspiration and one animal (LP4) had an ovulation within 3 days of aspiration without being detected in oestrus. One animal (HP4) developed a large anovulatory follicle ("cystic follicle") and was not detected in oestrus or detected to have an ovulation by Day 8 (HP4) and data from this animal were removed from all analyses. This left a total of 36 animals in the LP4 treatment (30 heifers and six cows) and 40 in the HP4 treatment (33 heifers and seven cows) group for analyses (Table 1). This also included 18 animals in the LP4 (eight heifers and one cow) and the HP4 (eight heifers and one cow) subgroups that were subjected to serial ultrasonography. One cow from the LP4-group and one heifer from the HP4-group were missing at the time of the final pregnancy test and were not included in the calculation of the 13-week pregnancy rate but were included in the assessment of values for other variables.

3.2. Concentrations of progesterone

Concentrations of progesterone in the subgroup of animals subjected to serial ultrasonography between Days 0 and 20 after follicular aspiration and which had ovulations by Day 9, were affected by treatment ($P = 0.006$), time ($P < 0.001$ and a treatment by time interaction was detected ($P < 0.001$). Concentrations of progesterone were greater on Days 2 and 4 following aspiration in the animals in the HP4-treatment group compared to those in the LP4-group (Fig. 2). On these days, concentrations of progesterone averaged 1.22 $\pm$ 0.11 ng/mL and 7.0 $\pm$ 0.60 ng/mL for animals in the LP4- and HP4-groups, respectively. On all other days in which concentrations of progesterone were assessed, mean concentrations did not differ between treatments.

When concentrations of progesterone in all of the treated animals were compared on Days 4 and 14 after follicular aspiration, a Mann-Whitney test indicated that concentrations were greater on Day 4 in animals in the HP4-treatment group compared to the LP4group (Fig. 3; $P < 0.001$) but were similar on Day 14 (Fig. 3; $P = 0.992$). The percentage of animals with concentrations of

Table 1

Characteristics of oestrus, pregnancy rates and the diameter of ovarian follicles in animals with either relatively lesser (LP4) and greater (HP4) concentrations of progesterone during development of potential pre-ovulatory follicles.

| Variable	LP4	HP4	P
n	36	40	
Age (years)	2.3 ± 0.13	2.3 ± 0.12	0.961
Weight (kg)	365.4 ± 9.3	364.2 ± 7.7	0.925
Body condition score (1-9)	5.2 ± 0.44	5.2 ± 0.41	0.825
Interval to oestrus (days)	6.1 ± 0.14	6.9 ± 0.10	< 0.001
Oestrous detection rate first AI (%)[a]	88.9 (32/36)	95.0 (38/40)	0.321
Ovulated by Day 14 (%)[b]	94.4 (34/36)	97.5 (39/40)	0.492
Ovulatory follicle at first AI (mm)[c]	12.1 ± 0.36	13.3 ± 0.27	0.021
Largest follicle at first AI (mm)	12.3 ± 0.55	12.8 ± 0.33	0.388
Pregnancy rate to first AI[d]	34.4 (11/32)	73.7 (28/38)	0.001
1-week pregnancy rate (%)	30.6 (11/36)	70.0 (28/40)	< 0.001
4-week pregnancy rate (%)	47.2 (17/36)	80.0 (34/40)	< 0.001
13-week pregnancy rate (%)[e]	80.0 (28/35)	94.9 (37/39)	0.051

[a] Animals detected in oestrus and inseminated/total number of animals.

[b] Percentage of animals with concentrations of progesterone in plasma > 1.5 ng/mL on Day 14 after follicular aspiration.

[c] Assessed in a subset of animals subjected to serial ultrasonography that ovulated after the first AI (LP4: $n = 7$; HP4: $n = 9$); results for other variables were assessed from all of the animals included in the study.

[d] Animals diagnosed as pregnant to the first AI/total number animals.

[e] One animal from each treatment group was missing at the time of the final pregnancy test.
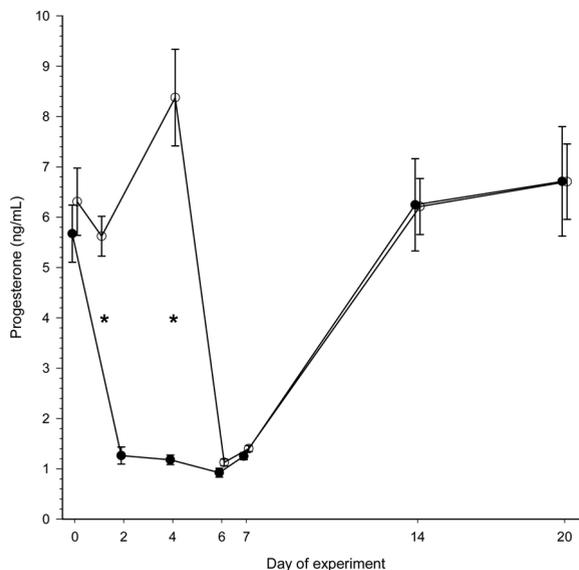
Fig. 2. Mean ± SEM concentrations of progesterone in a subset of animals between the day of follicular aspiration (Day 0) and Day 22 in which ovulation was detected; ● Animals treated with cloprostenol on Days 0 and 1 (LP4 treatment; $n = 7$); O animals treated with cloprostenol on Day 4 and 5 (HP4 treatment; $n = 9$); Effects due to treatment ($P = 0.006$), time ($P < 0.001$) and the interaction between treatment and time ($P < 0.001$); *Indicates where means differ ($P < 0.001$).

progesterone in plasma > 1.5 ng/mL on day 14 was similar between treatments (Table 1; $P = 0.472$).

3.3. Characteristics of oestrus and follicular development

There was no significant difference in the percentage of animals detected in oestrus within 9 days of aspiration (Table 1). The mean interval to oestrus was 0.8 days less in the animals in the LP4 compared to the HP4-treatment groups ($P < 0.001$, Table 1) but the synchrony of oestrus (homogeneity of variance) was similar ($P = 0.661$). No animals were detected in oestrus on Day 9. A total of 21 animals were detected in oestrus and inseminated between Days 19 and 29 (LP4: $n = 14$; HP4: $n = 7$). Further analyses were not conducted using these data as numbers were considered too small for analytical purposes with a high risk of committing a Type 1 or 2 statistical error. The average diameter of the largest follicle present in the ovary at the time of AI in all of the animals that were inseminated did not differ between treatment groups (Table 1). The diameter of the follicle from which ovulation occurred in the animals subjected to serial ultrasonography was, however, less in the animals in the LP4 compared to the HP4-treatment group ($P =$
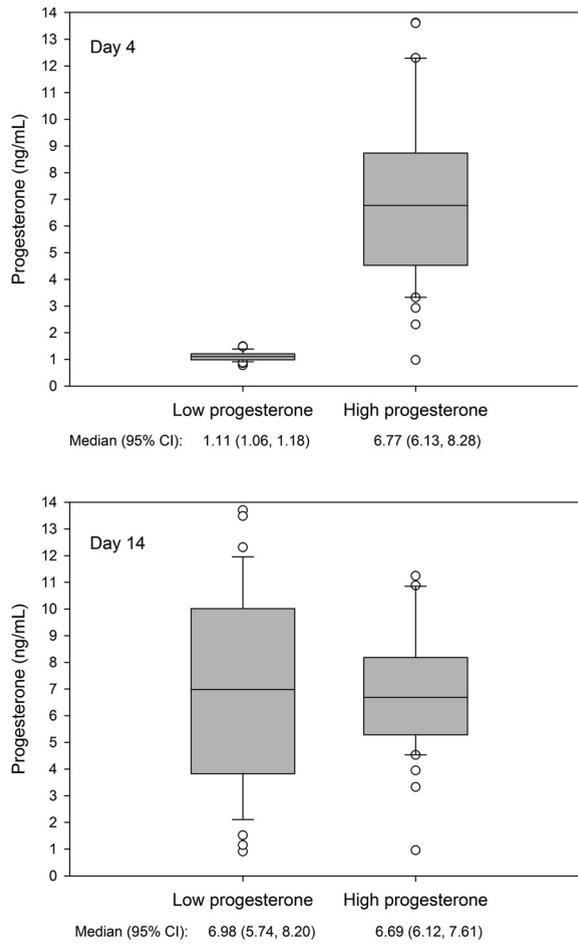
Fig. 3. Box and whisker plot of concentrations of progesterone in plasma 4 and 14 days after follicular aspiration in animals in the low (n = 36) and high (n = 40) progesterone treatments; Whiskers above and below the box indicate the 10th and 90th percentiles; Points above and below the whiskers indicate outliers outside the 10th and 90th percentiles; Median and 95% CI for each treatment are presented at the bottom outside each graph; Median concentrations differed on Day 4 ($P < 0.001$) but not on Day 14 ($P = 0.992$).
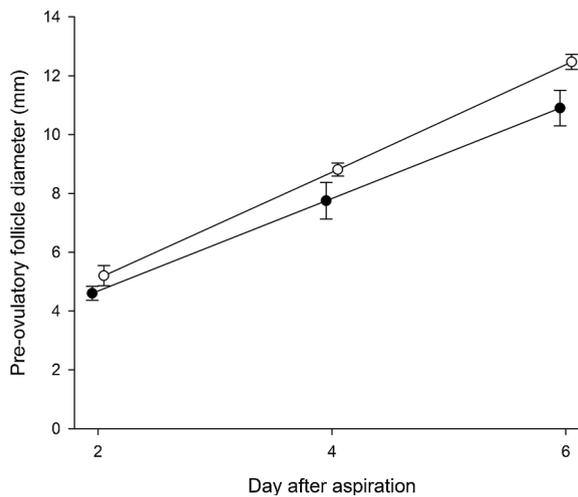
Fig. 4. Mean ± SEM diameter of the emerging pre-ovulatory follicle in animals with low (●) and high (O) circulating concentrations of progesterone between Days 0–4 after follicular aspiration; Effect of treatment ($P = 0.011$), time ($P < 0.001$) and treatment x time ($P = 0.475$).

Table 2

Results of logistic regression analysis of factors affecting submission rates to AI and pregnancy rates to AI in animals with either a relatively lesser (LP4) or greater (HP4) concentration of progesterone for 4 days following follicular aspiration.

Dependent variable	Factor	d.f.[a]	Coefficient	SE[a]	χ^2	P	Odds ratio (95% CI)[a]	Reference level
Oestrous detection rate – First AI	LP4	1	0.87	0.90	0.98	0.321	0.42 (0.07 – 2.45)	HP4
Ovulated by Day 14	LP4	1	−0.83	1.25	0.47	0.492	0.44 (0.04 – 5.02)	HP4
Pregnancy rate to first AI	LP4	1	−1.68	0.37	11.1	0.001	0.19 (0.07 – 0.52)	HP4
1-week pregnancy rate	LP4	1	−1.67	0.50	12.1	< 0.001	0.19 (0.07 – 0.50)	HP4
4-week pregnancy rate	LP4	1	−1.85	0.56	12.7	< 0.001	0.16 (0.05 – 0.47)	HP4
13-week pregnancy rate	LP4	1	−1.57	0.87	3.81	0.051	0.21 (0.04 – 1.15)	HP4
	Weight	1	0.03	0.01	6.40	0.011	1.03 (1.0 – 1.06)	

[a] d.f. = degrees of freedom; SE = standard error; CI = confidence interval.

0.021; Table 1).

The data for diameter of the emerging pre-ovulatory follicle in the animals that were subjected to serial ultrasonography and which had ovulations between Days 6 and 9 days after follicular aspiration (LP4: *n* = 7; HP4: *n* = 9), are depicted in Fig. 4. The diameter of the emerging pre-ovulatory follicle was greater in animals in the HP4-treatment compared to those in the LP4 treatment group between Days 2–6 (*P* = 0.011), it increased over time (*P* < 0.001) and no treatment x time interaction was detected (*P* = 0.475).

3.4. Pregnancy rates

Pregnancy rate to first AI and cumulative pregnancy rates at 1 and 4 weeks after the commencement of breeding were greater in animals in the HP4- compared to the LP4-treatment group. There was also a tendency for pregnancy rates to be less 13 weeks after follicular aspiration in the cows in the LP4-treatment group (Table 1). Age, whether animals were part of the serially ultrasonically assessed group or not, body condition score and weight did not affect outcome variables with the exception that the weight of animals did affect 13-week pregnancy rates (Table 2) with the probability of pregnancy increasing with increasing weight of the animals at the start of the study.

4. Discussion

The results of the present study highlight the potential for circulating concentrations of progesterone during early follicular development to affect fertility in spontaneously ovulating *Bos indicus* cattle. The presence or absence of a CL and resulting relatively lesser compared to greater circulating concentrations of progesterone during development of the pre-ovulatory follicle did not significantly affect the percentage of animals that were detected in oestrus and that had ovulations within 9 and 14 days of follicular aspiration, respectively or concentrations of progesterone after AI. It did, however, affect the odds of pregnancy to AI, with there being lesser concentrations of progesterone during the period of development of the pre-ovulatory follicle being associated with lesser fertility. These results support the conclusion that the fertility is affected by circulating concentrations of progesterone during the period of pre-ovulatory follicle development and that relatively greater basal concentrations of progesterone during follicular emergence are necessary for normal fertility.

The exact cause for the reduction in fertility in the present study that was associated with lesser concentrations of progesterone during development of the pre-ovulatory follicle could not be determined. Possible causes of the lesser fertility in the LP4-treated animals include changes in follicular growth patterns and/or intrafollicular concentrations of hormones leading to an alteration in oocyte viability (Revah and Butler, 1996; Inskeep, 2004; Cerri et al., 2011a), changes in luteal function and susceptibility to luteolysis (Fike et al., 1997; Cerri et al., 2011a) and endometrial morphology following ovulation (Shaham-Albalancy et al., 1997; Claro et al., 2010) or other unknown factors. The percentage of animals detected in oestrus after treatment, percentage that had ovulations by Day 14 and concentrations of progesterone in plasma 14 and 20 days after follicular aspiration did not differ between treatment groups in the present study. This suggests that the capacity of emerging follicles to acquire ovulatory capacity and produce sufficient oestradiol to induce behavioural signs of oestrus and form a functional CL was not affected by whether follicles emerged pre-ovulation when there was either relatively lesser or greater concentrations of progesterone. When there was relatively lesser concentrations of progesterone, but concentrations were greater than basal during treatment with progestins, there was an is associated increase in secretion of LH and oestradiol and prolonged duration of dominance of pre-ovulatory follicles (Kinder et al., 1996). This hormonal milieu can lead to disruption of gap junctions between granulosa and cumulus cells allowing for premature resumption of meiosis and germinal vesicle breakdown, resulting in an increase in embryonic mortality (Revah and Butler, 1996; Santos et al., 2016). In the present study, concentrations of progesterone were sufficiently less in the LP4-treatment group to enable developing follicles to progress to ovulation when mature so persistent states of dominance were unlikely. Thus, effects on oocytes may not have been the same as in studies where there has been examination of effects when persistent follicles developed in the presence of relatively lesser concentrations of progesterone. In previous studies, cows with relatively lesser compared to greater peripheral concentrations of progesterone had a greater frequency of LH pulses (Abreu et al., 2018a) and greater concentrations of oestradiol within follicles (Cerri et al., 2011a) and in plasma (Abreu et al., 2018a,b). There has been consideration that this may be detrimental

to fertility (Inskeep, 2004). Relatively lesser concentrations of progesterone, however, did not affect fertilisation rates and embryo quality in one study (Cerri et al., 2011b) or oocyte quality (Abreu et al., 2018a) or pregnancy rates (Abreu et al., 2018b; Martins et al., 2018) in other studies. Abreu et al. (2018b) suggested that *Bos taurus* cattle may be less sensitive to different circulating concentrations of progesterone, with sufficient gonadotropin stimulation for follicular development occurring across a wider range of concentrations compared with *Bos indicus* cattle.

In the current study, pre-ovulatory follicles in the LP4-treated animals developed in the absence of a CL or an exogenous source of progesterone which could be one reason why results of the present study differed from recent studies where there was a negative effect of relatively lesser circulating concentrations of progesterone on pregnancy rates to AI in *Bos taurus* cattle (Fricke et al., 2016; Abreu et al., 2018; Martins et al., 2018). In the studies conducted by Abreu et al. (2018a,b) and Martins et al. (2018), animals with relatively lesser concentrations of progesterone were treated with a used intravaginal progesterone releasing insert so that during development of follicles there were concentrations of progesterone that were greater than basal. Fricke et al. (2016) treated dairy cows using a double-Ovsynch protocol with cows having the relatively lesser progesterone treatment being treated with prostaglandin F2α 1 day after treatment with a third injection of GnRH that was aimed at inducing emergence of pre-ovulatory follicles. In this previous study, some animals could still have had an emerging CL during the period when pre-ovulatory follicles developed due to some corpora lutea being refractory to treatment with prostaglandin F2α when treated in the first 7 days following oestrus and ovulation (Braun et al., 1988). Pre-ovulatory follicles normally emerge during periods when there is a functional CL or progesterone before AI so investigation of fertility in one group of animals in the present study that lacked a CL during follicular development is atypical and may have contributed to differences in fertility observed. Emergence of pre-ovulatory follicles in the absence of a CL occurs during the normal transition from an anovulatory to an ovulatory state which occurs as animals transition from puberty and a postpartum period of anoestrus to undergoing regular ovulatory cycles. During these transitional phases fewer cows are expected to express oestrus, and ovulation is frequently followed by a short luteal phase (Kinder et al., 1995; Rhodes et al., 2003). Lactating cows at this time are also experiencing profound changes in energy balance, body condition, and metabolic hormones. In addition, both anovulatory and prepubertal females have changes in the hypothalamus and uterine morphology that are likely to influence fertility as they transition from anovulatory to a regularly ovulatory state (Day and Anderson, 1998; Rhodes et al., 2003; Atkins et al., 2013). Treatment of post-partum anovulatory cows with progesterone can improve expression of oestrus, decrease postpartum to ovulation intervals, increase pregnancy rates to AI and decrease early embryonic loss (Rhodes et al., 2003; Santos et al., 2016). In the present study, heifers and non-lactating cows were used that had ovulations prior to follicular aspiration in an attempt to study effects of concentrations of progesterone without physiological limitations associated with the post-partum period or transition to puberty. The results of the present study highlight that in oestrous cyclic females when there is development of pre-ovulatory follicles in the absence of a CL fertility is compromised. Unlike anovulatory and prepubertal females, expression of oestrus or concentrations of progesterone after ovulation were not affected, highlighting the possibility that oocyte viability was directly affected in the present study by the absence of a CL. Further investigation is required to determine what aspects of oocyte development and competence are disrupted during pre-ovulatory follicular development in the absence of an endogenous or exogenous source of progesterone.

When pre-ovulatory follicles emerge when there are lesser relative concentrations of progesterone there may be an increased risk of premature luteolysis occurring in a subsequent oestrous cycle (Fike et al., 1997). Cerri et al. (2011a) reported that the secretion of prostaglandin F2α in response to an oxytocin administration was increased in animals 16 days after induction of ovulation when pre-ovulatory follicles emerged when there was relatively lesser compared with greater circulating concentrations of progesterone. The incidence of short luteal phases also tended to increase (25% compared 0%; $P = 0.09$). Fricke et al. (2016), however, reported there was a greater mRNA abundance because of expression of five of six interferon-stimulated genes in dairy cows 18 days after a timed AI when pre-ovulatory follicles developed when there were relatively lesser concentrations of progesterone. Expression of such genes is likely to help prevent luteolysis so these findings do not support the concept that a lesser progesterone milieu during pre-ovulatory follicular development would always increase the risk of luteolysis. In the previous study, the main factor affecting concentrations of progesterone after treatment with relatively larger and smaller doses of progesterone was the size of the ovulatory follicle, with ovulatory follicles being larger in animals treated with a relatively lesser dose of progesterone. In the present study, diameters of the ovulatory follicles were smaller in the animals with the ultrasonic serial assessments that lacked a CL after follicular aspiration. This may have increased the risk of premature luteolysis (Fike et al., 1997) or sub-optimal concentrations of progesterone after ovulation occurring (Vasconcelos et al., 2001). Concentrations of progesterone in all animals on Days 14 and the serially sampled animals on Days 20, which equates to early and mid-dioestrus, respectively, did not, however, differ significantly in the present study. Thus, the function of a CL at these stages of the oestrous cycle did not appear to be compromised. Perhaps a longer duration or more intensive sampling was needed to detect differences in the function of corpora lutea or that detrimental effects on fertility in the present study were not mediated by effects on luteal function. Alternatively, as the largest follicle at AI in all of the treated animals did not differ between treatment groups, this could indicate that the differences in the diameter of the ovulatory follicle recorded in the animals subjected to serial ultrasonographic examination may not have been apparent if the diameter of the ovulatory follicle was recorded in all of the animals. It is possible that a greater number of animals were needed to assess potential differences in the diameters of ovulatory follicles in the present study. Other possible causes of differences in fertility between the LP4- and HP4-treated cattle in the present study could include changes in endometrial morphology and uterine diameter and tone which have been observed in dairy cows (Sham-Albalancy et al., 1997) and prepubertal heifers (Claro et al., 2010) with different circulating concentrations of progesterone before ovulation.

In the present study, the diameter of pre-ovulatory emerging follicles in animals that were subjected to serial ultrasonography were observed to be greater in animals that were in the HP4 compared to those in the LP4-treatment group (Fig. 4) while earlier onset of oestrus was detected in animals with relatively lesser peripheral concentrations of progesterone during follicular emergence.

Normally the diameters of growing pre-ovulatory follicles are expected to be greater in animals that have relatively lesser concentrations of progesterone due to the expected greater frequency of release of LH (Bergfeld et al., 1995, 1996). Earlier acquisition of ovulatory capacity in the animals in the LP4-treatment group in the present study is likely due to a greater pulsatile release of LH but there is uncertainty as to why the diameter of emerging follicles were smaller in the LP4-treated heifers between Days 2–6. Mean differences were small in the present study between treatment groups so perhaps differences could be attributed to a Type 1 error or perhaps the growth of follicles in animals where there was a lesser fertility may have been compromised to some extent. Ovarian follicular diameter at the time of AI in all of the animals that were treated did not differ significantly between treatment groups in the present study. It, therefore, cannot be ascertained in the present study if the follicular growth patterns observed in the ultrasonically assessed animals were representative of all of the animals and if there is a functional significance of the difference in the diameter of pre-ovulatory follicles that were observed in these ultrasonically assessed animals (Fig. 4).

In some previous studies where there was examination of effects of concentrations of progesterone on fertility a variety of variables may have had a potentially confounding effect on the fertility of oocytes. Potential confounding factors affecting the fertility of oocytes could include: induction of ovulation before follicles were fully mature (Lamb et al., 2001; Vasconcelos et al., 2001; Perry et al., 2005), animals being oestrous cyclic or acyclic before the start of treatment (Bisinotto et al., 2015b; Cavalieri et al., 2018), and the presence or absence of a functional CL at the time of initiating a timed AI protocol (Bisinotto et al., 2015b). New wave follicular emergence during experimental treatments may not have always occurred in some studies so the timing of progesterone supplementation in relation to when follicles emerged may have been misaligned in some animals. For example, response rates to an ovulation induction treatment with GnRH have been reported to be only 54% of heifers (Pursley et al., 1995), 66% of beef cattle (Geary et al., 2000) and 64%–85% of dairy cattle (Pursley et al., 1995; Vasconcelos et al., 1999). Thus, new wave follicular emergence may not have always occurred in studies where there was utilisation of GnRH. In the present study, there was assurance that there were ovulations in animals before commencing treatments, follicular aspiration was used to induce consistent emergence of a new follicular wave and only insemination of animals that spontaneously expressed signs of oestrus was undertaken in an attempt to control for potential confounding factors. Bisinotto et al. (2015b) suggested that improvements in pregnancy rates associated with supplementation with progesterone in dairy cattle were attributed to improvements in the synchronisation of oestrus which led to improvements in pregnancy rates to AI when cows are inseminated at a fixed time by reducing the proportion of cows having ovulations asynchronously with the time of AI. This perspective was supported by the finding in this previous study that pregnancy rates increased by only 3% when cattle were supplemented with progesterone and inseminated on detection of oestrus before the scheduled time of AI. In the present study, there were no differences in the extent of oestrous synchrony associated with pre-ovulatory follicular development in the presence or absence of a CL and all inseminations were completed after the detection of oestrus indicating that the cause of differences in fertility were not related to these factors. This suggests that progesterone has a fundamental function in oocyte development. Results of the present study, therefore, provide evidence without potential confounding effects that optimal concentrations of progesterone during development of the pre-ovulatory follicle are necessary to avoid a reduction in fertility. The present study was, however, conducted with a relatively small number of animals so further studies should be undertaken that do or do not corroborate results of this study.

Mean concentrations of progesterone between Days 2 to 4 in animals in the LP4-group that were subjected to serial sampling were 1.22 ± 0.11 ng/mL which is greater than concentrations normally expected after induction of luteolyis in most studies (Henricks et al., 1970; Rajamahendran et al., 1989) but comparable to mean concentrations of 1.3 ± 0.24 ng/mL recorded by Pinheiro et al. (1998) about 48 h after administering two doses of PGF2α 11 days apart in Nelore cattle that subsequently expressed behavioural signs of oestrus. Administration of two doses of prostaglandin F2α in the animals in the LP4 treatment after Day 6 of the oestrous cycle was expected to induce luteolysis and to reduce peripheral concentrations of progesterone to < 1.0 ng/mL (Henricks et al., 1970). The failure, in the present study, to reduce mean concentrations of progesterone to < 1.0 ng/mL in the animals in the LP4-treatment group that were blood sampled between Days 2 to 4 after follicular aspiration may have been due to non-specific binding from the presence of cross-reactive antigens or the antibody that was used for the assays had less affinity when compared with other assay systems used in other studies or the quantity of labelled tracer or antibody used in the assay was not optimal (Abraham, 1974).

5. Conclusion

When pre-ovulatory follicles emerged after follicular aspiration in *Bos indicus* cattle is lesser compared to greater concentrations of progesterone, the odds of pregnancy to AI and after 1, 4 and 13 weeks of breeding was less. This highlights the important aspect that concentrations of progesterone have during follicular development on fertility. These findings contribute to the evidence that it is a necessity to balance several factors when seeking to optimise fertility to AI which includes sufficient concentrations of progesterone during the period of development of pre-ovulatory follicles. Further studies are needed to elucidate the cause of low fertility in pre-ovulatory follicles that develop in a relatively lesser progesterone milieu without a functional CL but the results of the present study indicate that it was unrelated to the capacity of pre-ovulatory follicles to reach ovulatory capacity and to produce adequate concentrations of progesterone after ovulation.

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Conflicts of interest

The author declares no conflict of interest.

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