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Randomised controlled trial of the effect of concentration of progesterone before artificial insemination on fertility in ovulatory and anovulatory *Bos indicus* cattle

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Objective To investigate the effect of concentration of progesterone (P4) before artificial insemination (AI) on fertility in ovulatory or anovulatory *Bos indicus* cattle.

Design Randomised control study

Methods The study included 162 heifers and 96 lactating cows. On days –10 to –12, animals were examined using transrectal ultrasound, administered PG and examined for a corpus luteum (CL). Those with a CL were allocated to Experiment 1. On day 0 they were administered an intravaginal progesterone-releasing IVD (IVD) containing progesterone (P4) (0.78 g), oestrodiol benzoate (ODB) and either saline or PG to induce high and low circulating P4 concentrations, respectively. Those without a CL were re-examined on day 0 and those without a CL at both examinations were allocated to Experiment 2. Cows and heifers were treated with an IVD containing P4 at 0.78 g or 1.56 g to induce low and high P4 concentrations, respectively. IVDs were removed on day 7 and PG and equine chorionic gonadotrophin (eCG) were administered. Females in oestrus on day 9 were inseminated; others were administered ODB and inseminated 22–26 h later.

Results Greater concentrations of circulating P4 increased the odds of pregnancy to AI in anovulatory females (P = 0.008), but decreased the odds of pregnancy in one year but not another in ovulatory animals (P × year, P = 0.019).

Conclusion Manipulating P4 concentrations before AI has the potential to improve pregnancy outcomes to AI in *B. indicus* females, but treatment may need to vary between animals classified as anovulatory or ovulatory.

Keywords antral follicle count; artificial insemination; *Bos indicus*; fertility; progesterone; synchronisation

Abbreviations AFC, antral follicle count; AI, artificial insemination; CL, corpus luteum; eCG, equine chorionic gonadotrophin; IVD, intravaginal progesterone-releasing device; LH, luteinising hormone; ODB, oestradiol benzoate; P4, progesterone

The variable and often low fertility to artificial insemination (AI) is one factor that limits the use of AI in extensively managed beef herds where *Bos indicus* genotypes predominate. Fertility is also one of the key drivers of profitability in grazing enterprises in tropical environments.[1] It is dependent on multiple factors that affect the ability of cows to conceive and maintain pregnancies.[2] Some of these factors are physiological events that occur before, during and after ovulation, which can influence the establishment and maintenance of pregnancy.[3]

Concentrations of the hormone progesterone (P4) before and after ovulation are known to affect fertility by influencing both the quality of oocytes and embryonic development. In several studies, higher P4 concentrations before AI have increased fertility,[4–7] improved embryo quality and reduced the rate of early embryonic loss.[8] Low P4 concentrations before AI can reduce the number of follicular waves and increase the duration of follicular dominance, growth rates and maximum diameters of preovulatory follicles, which can negatively affect fertility.[9–11] Progesterone also appears to play a key role in oocyte maturation and appears to have an anti-apoptotic effect on oocytes and, therefore, could affect oocyte quality.[12] Low P4 concentrations before AI may also alter uterine function by promoting premature uterine synthesis of prostaglandin F2α, thus increasing the probability of luteolysis occurring prematurely within 16 days of AI.[13] In *B. indicus* cattle that have had their oestrous cycles synchronised, lower P4 concentrations before a timed AI has had a variable effect on fertility, with an increase in pregnancy rates being reported in some studies[14–17] but not others.[11,18,19] Thus more investigation of the effects of P4 concentrations before AI is needed.

Evidence is also accumulating that suggests the number of antral follicles ≥ 3 mm within the ovaries of dairy cows is positively associated with ovarian size,[20], fertility,[21] responses to superovulatory treatments[22–24] and P4 concentrations following oestrus.[25] There have been no reports, however, to date as to whether the number of antral follicles in *B. indicus* heifers or cows is associated with fertility in conventional breeding or AI programs. Evidence of such a relationship could provide the basis for selection of more fertile animals in the future.

The aim of this study was to determine the effects of altering P4 concentrations before AI on fertility in *B. indicus* heifers and cows with synchronised oestrous cycles and to determine if antral follicle count (AFC) had any association with fertility. Our first hypothesis was that higher circulating P4 concentrations during the emergence of a potential preovulatory follicle will improve pregnancy rates to AI in *B. indicus* females classified as ovulatory and anovulatory at the start of treatment. Our second hypothesis was that there would be a positive association between AFC prior to a synchronised ovulation and fertility.

Materials and methods

Approvals

The experimental procedures were approved by the James Cook University Animal Ethics Committee (Approval no. A1856).

Location of animals

The *B. indicus* (Brahman) heifers and cows were enrolled in the study between 2013 and 2015. In 2013 and 2015 the animals were located on pasture at the James Cook University Tropical Veterinary Research Station, Fletcherview, Queensland (latitude 19°53'4"S; longitude 146°10'43"E). In 2014 animals were maintained on pasture at the Department of Agriculture, Fisheries and Forestry Research Station, Swans Lagoon, Queensland (latitude 20°4’44’’S, longitude 147°13’27’’E; days –12 to 32 of the study) and after day 32 at the James Cook University Tropical Veterinary Research Station. Differences in location in 2014 occurred before and during the period of AI to enable increased dry matter intakes resulting from seasonally low rainfall. In 2015, the persistence of drought conditions required stock numbers to be reduced, so a number of animals (Experiment 1, n = 9; Experiment 2, n = 67) were not available for pregnancy diagnosis at the end of the study.

Treatments

We enrolled *B. indicus* heifers (n = 162), aged 20–22 months, and non-lactating (n = 66) and lactating (n = 96) cows aged between 3 and 9 years. Animals were initially weighed and had their ovaries examined by transrectal ultrasonography (Eureka SA-600, Medison; 7.5-MHz probe) to determine if a corpus luteum (CL) was visible (Figure 1). At this time the animals were administered 0.5 mg IM of PG (Estroplan®, Parnell Laboratories, NSW, Aust). On day 0, 10–12 days later, those animals without a CL detectable at the previous examination had their ovaries re-examined with ultrasound. Animals with a CL visible at either examination were classified as ovulatory and allocated to Experiment 1 in 2014 and 2015 and those without a CL at both examinations were classified as anovulatory and allocated to Experiment 2 in 2013 and 2015. In each experiment the animals were stratified by cycling status, type (heifer or cow) and bodyweight and then randomly allocated to one of two treatments.

In Experiment 1, ovulatory animals were treated on day 0 with an intravaginal P4-releasing device (IVD) containing 0.78 g of P4 (Cue-Mate, Bioniche Animal Health, NSW, Aust) and oestradiol benzoate (ODB: 1 mg/500 kg IM; Bomerol, Bayer Australia, NSW, Aust) was administered. Animals were then either administered 2 mL of 0.9% saline solution IM (saline, n = 95) or 0.5 mg of PG IM (PG, n = 86) to induce high and low circulating P4 concentrations, respectively. IVDs were removed 7 days later (day 7) and 0.5 mg of PG and 400 IU of eCG (Pregnecol, Bioniche Animal Health) were administered IM and aids for the detection of oestrus (Estrotect, Genetics Australia, VIC, Aust) were used. At 48 h after removal of IVDs (day 9), animals that were in oestrus (> 25% background colour of oestrous detection aid visible) were artificially inseminated with frozen and thawed semen. Animals that were not in oestrus were treated with ODB at the same time as the animals undergoing AI (1 mg/500 kg IM) and then inseminated 22–26 h later (Figure 1).

In Experiment 2, anovulatory animals were treated, on day 0, with an IVD containing 0.78 g (n = 70) or 1.56 g (n = 73) of P4 (Cue-Mate) to induce low and high P4 concentrations, respectively. Saline or PG were not administered as in Experiment 1 because these animals lacked a functional CL on day 0. ODB (1 mg/500 kg IM) was also administered on the same day (Figure 1). After day 0, treatments and AI were then identical to those applied to the ovulatory animals in Experiment 1, with IVDs being removed on day 7.

Ovarian ultrasonography and blood sampling

Transrectal ultrasonography of the ovaries of every animal in the study was conducted on either day 6 or 7 using a 7.5-MHz transducer (Mylab 5; Medical Plus Australia Pty Ltd, VIC, Aust) and at the time of AI in 2013. In 2015 a subset of 24 ovulatory heifers (n = 13, saline treatment; n = 11 PG treatment) were examined once daily from day 2 to when ovulation was detected. Video recordings of each ultrasound examination were made. All follicles ≥ 3 mm in diameter and CL were measured using electronic callipers and ovarian maps were drawn to record the diameter and number of follicles and CL present in each ovary.

Every animal in experiments 1 and 2 was blood sampled on day 7. Every animal enrolled in Experiment 1 in 2014 was also blood sampled on day 25. Blood samples were collected from the coccygeal vein or artery into heparinised tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes NJ, USA). After collection of blood, samples were immediately stored on ice until they could be centrifuged (2500*g*, 15 min). Plasma was then isolated and stored at –20°C until the time of assay.

AI and pregnancy diagnosis

AI was carried out using frozen-thawed semen by a single operator throughout the study. Bulls were placed with the herd 15 days later (day 25) and removed 12 weeks later in 2013 and 2014, and 10 weeks later in 2015. Pregnancy diagnosis was performed between days 56 and 61 with the aid of transrectal ultrasonography using a 7.5-MHz linear array transducer and again at least 5 weeks after removal of bulls.

Hormone assays

Concentrations of P4 in plasma in each experiment were determined using a radioimmunoassay kit (IBL P4 RIA, Abacus ALS, QLD, Aust). The minimum detectable limit of the assay was 0.10 ng/mL. For plasma pools containing 1.0, 5.9 and 9.3 ng/mL, the intra-assay coefficients of variation for each pool were 6.8%, 4.0% and 6.5%, respectively, and the interassay coefficients of variation were 10.7%, 13.3%, and 10.7%, respectively.

Statistical analysis

Analysis of variance was used to compare the number and diameter of ovarian follicles on day 6 or 7 between treatments, the P4 concentrations on day 7 and the diameter of the largest follicle on the ovary at the time of AI in heifers in 2013. Year, age (heifer or cow), treatment and all main interactions were included as factors in models where relevant and weight was included as a covariate when significant. When comparing the P4 concentrations on day 25 in 2014, treatment, age, whether animals were diagnosed pregnant to AI, the day of insemination and relevant interaction terms were included in the model. Each dependent variable was tested for normality using a Shapiro-Wilks test and examination of residual plots; homogeneity of variance was tested using Levine’s test. Data were transformed

($\log_{10}$) if necessary. Transformed dependent variables included the concentration of P4 at the time of removing IVDs and the diameter of the largest ovarian follicle at the end of treatment in Experiment 1. When undertaking a log transformation it is customary to back transform the mean values and report the geometric mean and a back transformed 95% confidence interval. Means, however, are presented as arithmetic means together with their standard error for consistency. In the case of AFCs, mean ± standard deviation (SD) is reported.

Multivariable logistic regression was used to model the effect of treatment or plasma concentration of P4 on day 7 on outcome variables (pregnancy rate to AI, submission rate on day 9 and final pregnancy rate) initially for each experiment separately. Submission rate was defined as the proportion of eligible cows and heifers that were submitted for AI up to day 9. Variables such as body weight, year, age (heifer or cow), bull, the total number of follicles ≥ 3 mm in diameter (AFC) and relevant interaction terms were included in initial models, where applicable, to assess potential associations with the outcomes. In the initial analyses, to avoid repetition of highly correlated variables, treatment and P4 concentrations on day 7 were used as explanatory variables in separate models because the variables were not independent of each other, with results published elsewhere for each study.[26] In this study the results of a combined analysis involving data from both experiments included only P4 concentrations on day 7 as the explanatory variable when it was found that treatment was not significantly associated with the dependent variables in either experiment. Terms were considered for elimination from each model using backwards step-wise logistic regression, although P4 concentrations on day 7 and the AFC were always left in their respective models. 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–Lemeshow test, while linearity of independent variables and log odds were tested using the Box–Tidwell transformation. 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 also calculated for all main effects remaining in models.

Statistical analyses were performed using the statistical software program IBM SPSS Statistics (version 20.0).

Results

Data exclusions

A total of four ovulatory animals and two anovulatory animals were excluded from all analyses because of incomplete data. At the time when the final pregnancy test was undertaken, a total of 82 animals (Experiment 1: 6 in 2014 and 9 in 2015; Experiment 2: 67 in 2015) were not available for testing and so were excluded from the analysis of final pregnancy rates. Data on AFCs were missing for one cow, which was excluded from analyses of AFCs but included in other analyses.

Experiment 1: concentrations of P4 and follicle size in ovulatory females

Concentrations of P4 on the day of removal of IVDs (day 7) were greater in animals treated with saline, compared with those treated with PG (8.92 ± 0.55 vs 4.14 ± 0.24; P < 0.001) and were less in 2014 compared with 2015 (5.68 ± 0.42 vs 6.85 vs 0.47 ng/mL, respectively; P < 0.001). In 2014 P4 concentrations were also greater in heifers compared with cows (6.50 ± 0.58 vs 4.65 ± 0.57, respectively), but in 2015 they were similar (6.75 ± 0.52 vs 7.17 ± 1.05; year by age interaction, P = 0.014). No other significant interactions were found (P > 0.30).
In 2014, on day 25, mean P4 concentrations did not differ significantly between treatments (P = 0.553), but were greater in animals that were pregnant to AI compared with those that were not (14.6 ± 0.83 ng/mL vs 9.5 ± 0.80 ng/mL, respectively; P < 0.001) and tended to be greater in cows compared with heifers (12.8 ± 0.83 ng/mL vs 10.9 ± 0.94 ng/mL, respectively P = 0.053). None of the interaction terms were significant (P > 0.290).
The diameter of the largest follicle imaged within the ovary on day 6 was less in animals treated with saline compared with those treated with PG (Table 1), less in heifers compared with cows (8.9 ± 0.24 mm vs 10.1 ± 0.40 mm, respectively; P = 0.017) and did not differ significantly between years (P = 0.467), and none of the interaction terms included in the model were significant (P > 0.30). Ovulation before AI was recorded in 28.6% (6/21) of the heifers in which follicle growth was monitored, with no significant differences between treatments being detected (15.4%, 2/13 vs 50.0%, 4/8, for saline and PG, respectively; P = 0.156). Pregnancy rates of those that were found to ovulate before and those that ovulated after AI did not differ (40.0%, 2/5 vs 60.0%, 9/15, respectively; P = 0.617).

Experiment 2: concentrations of P4 and follicle size in anovulatory females

In females enrolled in Experiment 2, the P4 concentrations on day 7 were greater in animals treated with a higher, compared with those treated with a lower dose of P4 (4.27 ± 0.16 ng/mL vs 3.39 ± 0.20 ng/mL, respectively; P = 0.001). Mean P4 concentrations were greater in heifers in 2013 compared with cows in 2015 (4.20 ± 0.27 ng/mL vs 3.63 ± 0.14 ng/mL, respectively; P = 0.040). A treatment by age (heifer or cow) interaction was not detected (P = 0.626).

The diameter of the largest follicle in the ovary on day 6 or 7 was greater in animals treated with a lower compared with a higher dose of P4 (9.4 ± 0.27 mm vs 8.8 ± 0.25 mm; P = 0.039; Table 1) and was greater in heifers in 2013 compared with cows in 2015 (9.7 ± 0.28 mm vs 8.9 ± 0.27 mm, respectively; P = 0.017). Weight was included in the model as a significant covariate (P = 0.008). The interaction between treatment and age was not significant (P = 0.538).

In heifers in 2013, the mean diameter of the largest ovarian follicle at the time of AI was greater in heifers treated with a lower compared with higher dose of P4 (13.0 ± 0.63 mm vs 11.0 ± 0.49 mm, respectively; P = 0.016). In 2013 more heifers that were treated with a lower compared with a higher dose of P4 ovulated before the time of AI (41.7%, 10/24 vs 14.8%, 4/27, respectively; P = 0.032). Pregnancy rates to AI were lower in those heifers that had ovulated, compared with those that had not ovulated by the time of AI (42.9%, 6/14 vs 75.7%, 28/37, respectively; P = 0.027).

Analysis of merged data

Submission rate day 9 for all females. The odds that animals were submitted for AI 2 days after removal of IVDs was greater in ovulatory compared with anovulatory animals (46.2%, 84/182 vs 21.4%, 30/140, respectively; P < 0.001), in animals with lower P4 concentrations at the time of removing IVDs (P < 0.001) and in animals with follicles that were larger in diameter on day 6 or 7 (P < 0.001; Table 2, Figure 2). *Pregnancy rates in experiments 1 and 2.*When pregnancy rates in the ovulatory and anovulatory animals were analysed separately, no significant effects of treatments were found (P > 0.15; Table 1). Data were, therefore, combined for the anovulatory and ovulatory females, ensuring that variables such as cycling status, P4 concentrations on day 7 and any relevant explanatory variables were included in the multivariable logistic regression models.

The odds of animals being diagnosed as pregnant to AI were greater in ovulatory compared with anovulatory animals (48.6%, 86/177 vs 45.4%, 64/141, respectively; P = 0.008; Table 2) and varied between years, being greater in 2013 than in 2015 but not between 2014 and other years (66.7%, 34/51, 46.8%, 44/94, 41.6%, 72/173, for 2013–2015, respectively; P = 0.007; Table 2). Pregnancy rates to AI were affected by interactions between the concentration of P4 at the time of removal of IVDs and whether animals were ovulatory or not at the start of treatment (P = 0.024) and by an interaction between the concentration of P4 at the time of removal of IVDs and year (P = 0.019; Table 2). Concentrations of P4 at the time of removal of IVDs were positively associated with pregnancy in the anovulatory but not the ovulatory animals. In 2014, lower P4 concentrations at the time of removal of IVDs were associated with significantly greater odds of pregnancy (Figure 3).

AFC was not significantly associated with pregnancy rates to AI when entered as a continuous variable (Table 2), but if entered as a categorical variable with females classified as having AFC ≤ 4 or > 4, there was a tendency for pregnancy rates to AI to be less in females with AFC ≤ 4 compared with those with AFC > 4 (31.4%, 16/51 vs 50.4%, 134/266, respectively; model P = 0.081).

Final pregnancy rates were not significantly affected by the concentration of P4 at the time of removal of IVDs, but an interaction between concentration of P4 and year was detected (P = 0.031; Table 2). In 2014, final pregnancy rates were positively associated with lower P4 concentrations at the time of removal of IVDs. Final pregnancy rates were also lower in anovulatory compared with ovulatory animals (64.9%; 48/74 vs 85.2%, 138/162, respectively; P < 0.001) and were greater in 2014 compared with 2015 (80.4%, 41/51; 89.8%, 79/88; 68.0%, 66/97; for years 2013–2015, respectively; P = 0.031, Table 2). No significant association between AFC and final pregnancy rates was found when AFC was entered as either a continuous (P = 0.975) or categorical variable (71.9%, 23/32 vs 79.9%, 163/204 for females with AFCs ≤ 4 and > 4, respectively; P = 0.929). Mean (± SD) AFC was 7.6 ± 3.2 (range, 1–20).

Discussion

Optimising fertility to AI when synchronising oestrus is dependent on animal, environmental and physiological factors.[27] The results of this study highlighted that responses to the synchronisation protocols used in this study varied across years, treatments, cycling status and age. This highlights the complexity associated with optimising pregnancy rates to treatments across herds and how variations in treatment protocols that might cause differences in P4 concentrations can yield differences in responses. Variability in pregnancy rates to AI in *B. indicus* females reduces the benefits of AI within beef cattle in tropical environments. The results of this study suggested that increasing the percentage of animals cycling before starting treatments, increasing P4 concentrations in anovulatory animals, decreasing P4 concentrations in ovulatory animals and possibly eliminating animals with very low AFC may be factors that could reduce variability in the response to treatments.

Ovarian follicle size

In the final analysis, lower circulating P4 concentrations at the end of treatment with an IVD was associated with greater diameters of the largest ovarian follicle on day 6 or 7 in both ovulatory and anovulatory females and greater odds of submission to AI 48 h after removing the IVD. These findings are consistent with the results of most[11,14,16,17] but not all studies[15] that have shown that when circulating P4 concentrations are lower larger ovarian follicles develop during synchronisation treatments. Other studies have also demonstrated an inverse relationship between the diameter of dominant follicles at the time of inducing luteolysis and the interval to the onset of oestrus.[28] Larger follicles towards the end of treatment in animals with lower circulating P4 concentrations would explain why the odds of submission to AI on day 9 were greater in animals with lower P4 concentrations. Differences in emerging follicle diameters when synchronising oestrus with progestogens and oestradiol have been suggested to be caused by P4-mediated suppression if luteinising hormone (LH) secretion reducing growth rates and dominant follicle diameters during follicular development in females with greater circulating P4 concentrations.[16,17] Recent data from our group[26] and others,[29] however, suggests that a delay in new wave emergence rather than differences in follicular growth rates may occur when *B. indicus* females are treated with higher doses of P4 in combination with ODB. This may contribute to differences in emerging follicle size when applying treatments that synchronise oestrus.

Fertility

We found that pregnancy rates to AI were variable, with lower P4 concentrations decreasing the odds of pregnancy in the anovulatory animals and increasing the odds of pregnancy to AI in ovulatory animals in 2014 but not in 2015. These results suggested that anovulatory animals may benefit from higher doses of P4 when synchronising oestrus, but in ovulatory animals, response can vary between years, with a beneficial effect of lower circulating P4 concentrations being evident in some years but not others. The reasons for differences between years were not apparent from the results of this study.

Variation in pregnancy rates to AI following treatments that induce differences in circulating P4 concentrations have been observed in other studies. One study recorded greater pregnancy rates to a timed AI when mean concentrations of serum P4 were 2.0 ng/mL (53.2%, 42/79) compared with heifers that had a mean P4 concentration of 2.3 ng/mL (37.8%, 28/74) or 3.0 ng/mL (37.2%, 29/78; $P < 0.10$).[14] A negative association between P4 concentration during treatment with an IVD and pregnancy rates was also reported in cycling *B. indicus* cows[15] and postpubertal *B. indicus* heifers[17] undergoing timed AI. A meta-analysis of 25 studies, involving over 16,000 dairy cows, found that P4 supplementation using an IVD significantly increased pregnancy rates to AI by between 3% and 4% between 32 and 60 days after AI.[7] Significant increases in pregnancy rates were, however, confined to cows that did not have a CL at the start of the synchronisation treatment or underwent timed insemination without the detection of oestrus. One study found that P4 supplementation as part of an Ovsynch protocol reduced pregnancy rates to AI in cows that were inseminated at a set time and which maintained their CL until administration of prostaglandin F2α (40.3% vs 46.7%),[30] but reported increased pregnancy rates to AI in those that did not have a CL at that time (38.1% vs 27.7%). Other studies have failed to detect differences in pregnancy rates when cattle with synchronised oestrous cycles have been exposed to higher P4 concentrations before ovulation.[11,18,19] When anoestrous dairy cows were administered a single IVD, containing 1.56 g of P4 or a modified IVD containing 4.7 g of P4, plasma P4 concentrations increased during treatment but pregnancy rates were not significantly affected.[31] These studies support the view that pregnancy rates are not always improved with greater P4 concentrations before AI in ovulatory or anovulatory females. They support our findings that greater P4 concentrations before AI may, at times, be more beneficial to females that are anovulatory at the start of a synchronisation treatment and lower P4 concentrations before AI may promote better pregnancy rates in animals that are ovulatory at the start of treatment.

A lack of consistency in fertility responses in studies where P4 concentrations have varied before AI could be for several reasons. For example, a failure to induce differences in circulating P4 concentrations between treatments was observed in one study.[11] Adequate secretion of LH to support follicular development during treatment in females with greater P4 concentrations might have occurred in some studies, resulting in adequate follicular maturity at the time of AI.[18] Administration of prostaglandin F2α before removing P4-releasing IVDs may have counteracted any potential negative influences of high P4 concentrations on follicular development in some studies, as this would induce lower the P4 concentration towards the end of treatment, which would stimulate an increase in the pulsatile release of LH, thus stimulating follicles to reach an adequate size before ovulation.[15] In studies that have used GnRH-based protocols, greater P4 concentrations before a timed AI could have improved pregnancy rates to AI, through indirect means such as reducing the number of animals entering oestrus and ovulating before a scheduled AI, improving the synchronisation of oestrus and reduced pregnancy loss rates after AI.[7] In this study, the insemination strategy involved inseminating females that were spontaneously in oestrus at 48 h after removing IVDs and delaying timed AI to at least 72 h for females that were not detected in oestrus at 48 h. This may have counteracted some of the potential negative influences that a high concentration of P4 might have on preovulatory follicular development by allowing females with smaller follicles at the time of IVD removal, which were not in oestrus at 48 h after removing IVDs, more time for preovulatory follicles to increase in maturity before AI occurred. Administration of equine chorionic gonadotrophin (eCG) at the time of removal of IVDs has also been shown to increase follicle diameter, ovulation rates and fertility in *B. indicus* cattle at timed AI.[15,17,32] Administration of eCG may also have reduced some of the potential negative effects that high P4 concentration had on fertility in some studies, by stimulating follicular development between the time of IVD removal and AI. Thus factors that could contribute to a lack of consistency in fertility responses associated with increasing P4 concentrations before the time of AI include adequate follicular development in the face of elevated P4 concentrations, variation in the timing and application of other treatments and the insemination strategy.

Benefits associated with exposure to P4 before ovulation have included increases in synchronised ovulation rates,[33,34] improvement in oocyte quality,[12] increased binding of gonadotrophin to LH receptors,[35] alteration in the expression of angiogenic factors in large preovulatory follicles, which improves subsequent luteal development and function,[36] and prevention of premature release of prostaglandin F2α following ovulation.[13] Improvement in fertility in anoestrous animals with greater P4 concentrations during a synchronisation treatment may suggest there is an optimal circulating concentration in these animals that facilitates the beneficial effects of P4. The inconsistent responses in ovulatory animals may also suggest that emerging follicles are responsive and able to grow at an adequate rate with different P4 concentrations but in some years fertility may be impeded when P4 concentrations are higher.

Our lower pregnancy rates in the anovulatory compared with the ovulatory animals is similar the findings in other studies.[37–39] Numerically, pregnancy rates to AI were, however, only approximately 3% greater in the ovulatory animals (48.6%, 86/177 vs 45.4%, 64/141), suggesting that the treatments used in anovulatory animals were successful in achieving acceptable pregnancy rates in a class of animal in which delayed conception and, as a consequence reduced economic returns would be expected if only natural breeding had been used. Physiological causes of reduced pregnancy rates in animals that are anoestrous at the start of synchronisation treatments include imprecise synchronisation reducing fertilisation rates,[40] failure of treatment to induce ovulation and increased rates of embryonic loss.[38] It is likely that a combination of these factors was responsible for the reduction in pregnancy rates in the anovulatory animals in this study.

Ovulation before AI

Ovulation before AI also appeared to contribute to the variability in pregnancy rates in our study. A greater percentage of heifers in Experiment 2 in 2013 that were treated with lower P4 concentrations ovulated before AI (41.7% vs 14.8%). Heifers that ovulated before AI also had lower pregnancy rates to AI. In Experiment 1, the percentage of heifers that ovulated before AI was 34.6% higher in heifers treated with PG, but statistical power was low and differences were not significant. Failure to detect oestrus or the occurrence of a preovulatory LH surge without expression of signs of behavioural oestrus in some females in this study could explain why some animals ovulated before AI. This suggests that detection of oestrus and AI may need to occur even earlier or at more frequent intervals after IVD removal, especially when lower circulating P4 concentrations exist during treatment with an IVD. Additional methods may also need to be used to increase the sensitivity of detection of oestrus.

Antral follicle count

AFC was not significantly associated with pregnancy rates to AI or at the end of the breeding period when entered into statistical models as a continuous variable. When entered as a categorical variable, pregnancy rates to AI tended to be greater in females with AFC > 4. Mossa et al.[21] reported that cows with AFC < 15 had lower pregnancy rates to first service compared with cows that had AFCs between 16 and 24 and lower odds of pregnancy at the end of the breeding season compared with cows with AFC ≥ 25. In this study, final pregnancy rates in females with AFC > 4 were 8% greater than those with AFC < 4, but differences were not significant. These results suggested that pregnancy rates to AI using the treatment and insemination strategies applied in this study may be compromised in females with very low AFCs, but that some recovery in fertility may occur by the end of the breeding season in animals with low AFCs at the time of AI. Feeding diets that increase circulating concentrations of insulin can increase follicle numbers in cows,[41,42] so AFCs may have improved by the end of the breeding season, when any nutritional limitations on ovarian function were expected to be less in the environment in which this study was undertaken. Differences in the magnitude of the AFC and associated effects on fertility between studies could be related to differences between breeds, diets, the methods used to determine AFC, differences in statistical power and treatments given before AI. Further study with more animals will be needed to determine if animals with very low AFCs consistently show lower fertility over time or if any effect of low AFC on fertility in *Bos indicus* cattle may reflect nutritional or genetic differences.

Study limitations

Differences in environments, rainfall patterns between years, animals, the length of the period of natural mating and removal of some animals before a final pregnancy test could be conducted may have contributed to some of the differences observed between years in this study. Initial analyses of each experiment allowed treatment to be excluded as a significant factor that affected pregnancy rates and allowed the combining of data from both experiments. Effects of year and age (heifer or cow) were confounded when comparing results between some years, so assessment of the results should include an understanding that any differences detected between years could have been attributed to either differences in ages, years or both. Further study with larger groups of animals is needed to improve statistical power and to validate the findings of this study.

Conclusions

We identified a number of potential sources of variation in pregnancy rates in *B. indicus* cattle with synchronised oestrous cycles. Greater mean P4 concentrations during new wave emergence before a synchronised oestrus and ovulation increased the odds of pregnancy in animals that were anovulatory at the start of treatment. In ovulatory animals, results were variable, with lower P4 concentrations favouring higher pregnancy rates in some years but not in others. Submission rates at 48 h after removal of IVDs and pregnancy rates to AI were also lower in animals classified as anovulatory compared with ovulatory at the start of treatment. Females with a very low AFC (≤ 4) tended to have lower pregnancy rates to AI. Variability in pregnancy rates and responses to different circulating P4 concentrations in *B. indicus* females suggested that different treatment strategies may be needed in females that are ovulatory or anovulatory at the start of treatments to optimise pregnancy rates.

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Conflicts of interest and sources of funding and

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Figure 1. Flow chart to show the treatment protocol used.
AI, artificial insemination; BS, P4, progesterone; PG, cloprostenol; ODB, oestradiol benzoate; ecG, equine chorionic gonadotrophin.

Figure 2. Fitted curve of the relationship between (a) the concentration of progesterone (ng/mL) at the time of removing intravaginal progesterone-containing devices (IVDs) (day 7) and (b) the diameter of the largest follicle imaged in the ovary on day 6 or 7 and the probability of submission to artificial insemination (AI) on day 9 (P < 0.001).

Figure 3. Fitted curves of the relationship between the concentration of progesterone (ng/mL) at the time of removing intravaginal progesterone-containing devices (IVDs) (day 7) and the probability of pregnancy to artificial insemination (AI) in (a) anovulatory females and (b) ovulatory females in 2014 ($P < 0.05$).

Table 1. The number of *Bos indicus* animals assigned to different treatments in experiments 1 and 2, diameter of the largest follicle imaged in the ovary on day 6 or 7, percentage submissions to artificial insemination (AI) on day 9 and percentage pregnant

Variable	Year	Cycling status			
		1: ovulatory		2: anovulatory	
Treatment		Saline	PG	0.78 g of P4	1.56 g of P4
n	2013	-	-	24	27
	2014	49	45		
	2015	43	40	45	45
	2013–15	92	85	69	72
Weight (kg)	2013	-	-	293.7 ± 3.6	293.5 ± 4.5
	2014	380.8 ± 5.2	388.5 ± 6.2	-	-
	2015	326.6 ± 4.5	324.7 ± 4.3	362.5 ± 5.8	364.6 ± 5.4
	2013–15	355.2 ± 4.5	358.5 ± 5.2	338.6 ± 5.6	337.9 ± 5.5
BCS (1–9)	2013	-	-	4.0 ± 0.04	4.1 ± 0.03
	2014	4.6 ± 0.06	4.5 ±0.07	-	-
	2015	4.0 ± 0.07	4.1 ± 0.07	-	-
	2013–15	4.3 ± 0.05	4.3 ± 0.05	-	-
Follicle diameter day 6 or 7 (mm)	2013	-	-	9.8 ± 0.37^{e}	8.8 ± 0.38^{f}
	2014	8.2 ± 0.31^{A}	11.2 ± 0.50^{B}	-	-
	2015	7.6 ± 0.28^{A}	10.4 ± 0.36^{B}	9.3 ± 0.36	8.8 ± 0.34
	2013–15	7.9 ± 0.21^{A}	10.8 ± 0.31^{B}	9.4 ± 0.26^{C}	8.8 ± 0.25^{D}
Percent submitted for AI on day 9 (n)	2013	-	-	12.5 (3/24)	25.9 (7/27)
	2014	28.6 (49)^{A}	73.3 (45)^{B}	-	-
	2015	7.0 (43)^{A}	75.0 (40)^{B}	33.3 (45)^{C}	13.3 (45)^{D}
	2013–15	18.5 (92)^{A}	74.1 (85)^{B}	26.1 (69)	18.1 (72)
Percent pregnant to AI (n)	2013	-	-	58.3 (24)	74.1 (27)
	2014	40.8 (49)	53.3 (45)	-	-
	2015	51.2 (43)	50.0 (40)	31.1 (45)	35.6 (45)
	2013–15	45.7 (92)	51.8 (85)	40.6 (69)	50.0 (72)
Final Percent pregnant (n)	2013	-	-	79.2 (24)	81.5 (27)

	2014	84.8 (46)	95.2 (42)	-	-
	2015	78.4 (37)	81.1 (37)	30.8 (13)	30.0 (10)
	2013–15	81.9 (83)	88.6 (79)	62.2 (37)	67.6 (37)

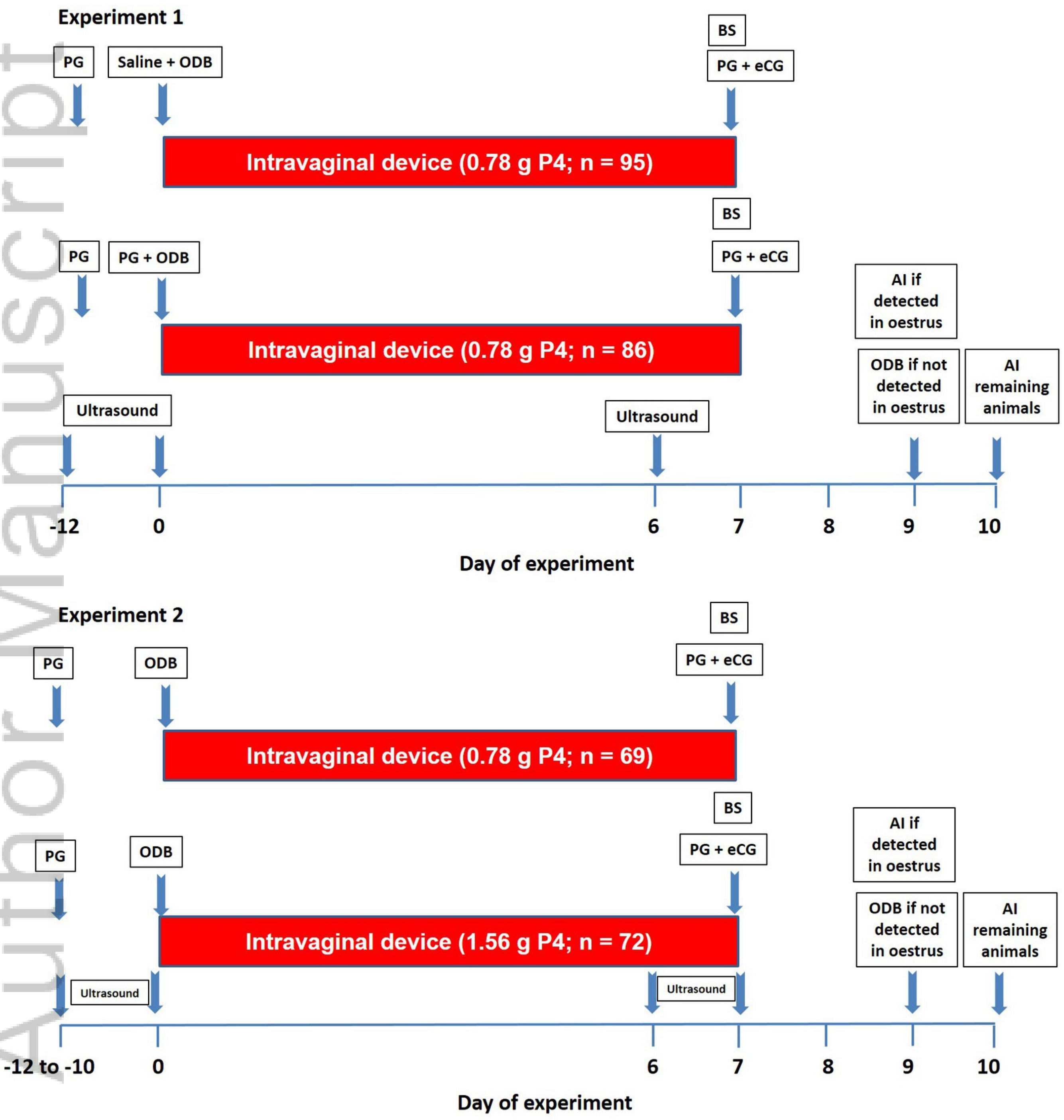
Means within rows, within an experiment differ at (P < 0.001)^{AB} or (P < 0.05)^{CD} or (P < 0.10)^{EF}.

AI, artificial insemination; BCS, body condition score; P4, progesterone.

Table 2. Multivariable logistic regression analysis of the combined data from experiments 1 and 2 for variables found to significantly affect submission rate to AI on day 9, having a follicle ≥ 9 mm in diameter on day 6 or 7 and pregnancy rate to artificial insemination in *Bos indicus* cows and heifers

Dependent variable	Explanatory variable	df	P value	Odds ratio (95% CI[a])	Reference group
Submission rate day 9	Anovulatory	1	<0.001	0.17 (0.09–0.30)	Ovulatory
	Follicle diameter day 6 or 7	1	<0.001	1.40 (01.2–1.6)	-
	P4	1	<0.001	0.72 (0.64–0.82)	-
Pregnancy rate to AI	P4 at removal	1	0.559	1.02 (0.95–1.1)	-
	Anovulatory	1	0.008	0.11 (0.02–0.59)	Ovulatory
	AFC	1	0.262	1.0 (0.96–1.1)	-
	Year	2	0.007		
	Year 2013	1	-	17.8 (2.3–138.3)	2015
	Year 2014	1	-	2.3 (0.78–6.8)	2015
	P4 at removal × cycling status	1	0.024	1.5 (1.0–2.2)	-
	P4 at removal × year	2	0.019	-	-
	P4 × year (2013)	1	-	0.66 (0.41–1.1)	2015
	P4 × year (2014)	1	-	0.85 (0.74–0.98)	2015
Final pregnancy rate	P4 at removal	1	0.335	1.1 (0.94–1.2)	-
	Anovulatory	1	<0.001	0.14 (0.04–0.42)	Ovulatory
	AFC	1	0.975	1.0 (0.89–1.1)	-
	Year	2	<0.001		
	Year 2013	1		9.6 (3.1–29.9)	2015
	Year 2014	1		2.3 (0.89–1.1)	2015
	P4 at removal × year	2	0.031		
	P4 × year (2013)	1		0.75 (0.53–1.1)	2015
	P4 × year (2014)	1		0.78 (0.63–0.97)	2015

[a] df. AFC, Antral follicle count; CI, confidence interval; df, degrees of freedom; P4, progesterone.



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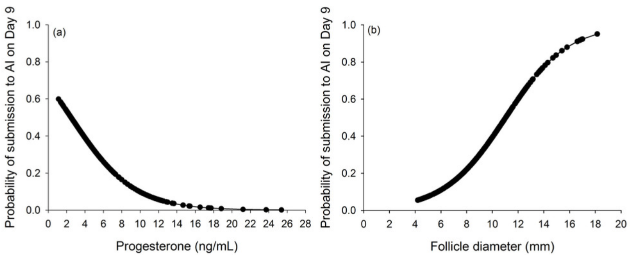

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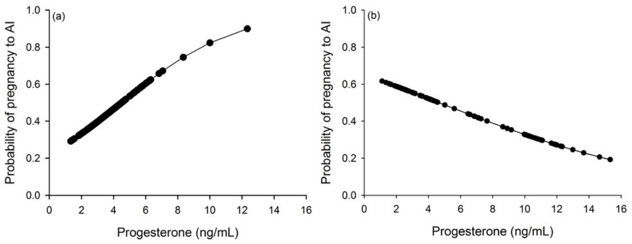

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