final report

Project code: B NBP.0760

Prepared by: John Cavalieri
James Cook University

Date published: 21 December 2015

ISBN: MLA TO PROVIDE

PUBLISHED BY
Meat and Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Improving fertility to AI in Bos indicus cattle

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Increasing circulating concentrations of progesterone before AI has been shown to improve pregnancy rates to AI, but reduced follicle diameters and pregnancy rates to timed AI in some studies. Fertility in dairy cows has also been positively associated with antral follicle counts (AFC's). Three experiments were conducted in *Bos indicus* heifers and cows to determine if increasing circulating concentrations of progesterone during preovulatory follicular development or differences in AFC's could affect fertility to AI. Greater circulating concentrations of progesterone increased the odds of pregnancy to AI in anovulatory females but had variable effects on fertility in ovulatory animals, decreasing the odds of pregnancy in one year but not another. Treatment with greater doses of progesterone in combination with administration of oestradiol benzoate delayed new wave emergence and reduced the diameter of emerging follicles when treatment with exogenous progesterone ended but did not significantly affect the growth rate of emerging follicles. Pregnancy rates to AI tended to be less in females with AFC's ≤ 4 compared to those with AFC's >4 . Manipulating concentrations of progesterone and AFC before AI has the potential to improve pregnancy outcomes to AI in *Bos indicus* females.

Executive Summary

Variable and often low fertility to AI in *Bos indicus* females is one factor that limits the use of AI in extensively managed beef herds. Increasing concentrations of progesterone during preovulatory follicular development has been shown to improve oocyte quality and pregnancy rates to AI in some studies. The number of follicles ≥ 3 mm in diameter in the ovaries of dairy cows (antral follicle count; AFC) has also been positively associated with fertility. The aim of these studies was to determine if manipulating circulating concentrations of progesterone during preovulatory follicular development or differences in antral follicle counts could affect fertility to AI in *Bos indicus* females.

Bos indicus heifers and cows were allocated to three experiments from 2013 to 2015. Between Days -12 and -10 animals were examined with transrectal ultrasonography for the presence of a corpus luteum (CL) and administered cloprostenol (0.5 mg IM). Animals without a CL detectable on the first examination were re-examined with ultrasound on Day 0. Animals with a CL (ovulatory) were allocated to Experiment 1 in 2014 and 2015 while animals without a CL at both examinations (anovulatory) were allocated to Experiment 2 in 2013 or 2015. In a separate study anovulatory animals were also allocated to Experiment 3 in 2013. In Experiment 1 animals were administered an intravaginal device (IVD) containing 0.78 g of progesterone, oestradiol benzoate (1 mg/500 kg) on Day 0 and then either administered saline (Saline) or cloprostenol (PG) intramuscularly. Animals were subjected to transrectal ultrasonography on Day 6 and a subset of animals between Days 0 and ovulation. On Day 7 a blood sample was taken, IVD's were removed and cloprostenol (0.5 mg IM) and equine chorionic gonadotrophin (eCG; 400 IU IM) were administered. Animals detected in oestrus on Day 9 were artificially inseminated and the remaining animals were administered oestradiol benzoate (1 mg/500 kg IM) and inseminated 22 to 26 hours later. In Experiment 2 animals received the same treatment except an IVD containing either 0.78 g or 1.56 g of progesterone was administered and ultrasound was performed on either Day 6 (2015) or 7 (2013). In Experiment 3 anovulatory heifers were treated with two IVD's containing either 0.78 g, 1.56 g, 2.34 g or 3.12 g of progesterone. Devices were inserted on Day 0 concurrently with administration of oestradiol benzoate (1 mg/500 kg IM) and removed on Day 7 when eCG (400 IU IM) was administered. Forty eight hours after removal of inserts, animals that were not detected in oestrus were treated with oestradiol benzoate (1 mg/500 kg IM and were not inseminated.

In Experiments 1, females treated with Saline compared with PG, had mean concentrations of progesterone greater on Day 7; later emergence of follicles that were classified as dominant on Day 8 (Day 3.8 ± 0.33 versus 2.4 ± 0.42, P = 0.016) and the maximum follicle diameter was less on Day 6 (7.9 ± 0.21 mm versus 10.8 ± 0.31 mm; $P < 0.05$). Pregnancy rates to AI and at the end of the breeding seasons did not, differ significantly between treatments. Odds of pregnancy to AI were greater in 2014 in animals with lower concentrations of progesterone on Day 7 but not in 2015. In Experiment 2, pregnancy rates of females treated with 0.78 g compared with 1.56 g of progesterone were not affected by treatment but the odds of pregnancy to AI were increased in females with greater concentrations of progesterone on Day 7 ($P = 0.045$). In Experiment 3 different doses of progesterone did not significantly affect the growth rates of emerging follicles. Follicle diameters were greater in heifers treated with 0.78 g compared with 1.56 g of progesterone between Days 6 and 10. Mean follicle diameters did not differ significantly between other

treatments. Growth rates ($P = 0.021$) and mean diameters ($P < 0.001$) of emerging follicles were greater in follicles that did ovulate compared to those that did not. When data from Experiments 1 and 2 were combined pregnancy rates were greater in ovulatory compared to anovulatory animals. Antral follicle count was not significantly associated with any pregnancy outcomes. When treated as a categorical variable pregnancy rates to AI tended to be less in females with AFC's ≤ 4 compared to those > 4.

When treating *Bos indicus* females with an IVD and oestradiol benzoate to synchronise preovulatory follicular development, greater concentrations of progesterone can increase pregnancy rates to AI in anovulatory animals while in ovulatory females pregnancy rates are increased in some years when lower concentrations of progesterone occur. Differences in circulating concentrations of progesterone can affect preovulatory follicular diameters by altering the day of emergence relative to the start of treatment with progesterone and oestradiol. This could affect pregnancy rates when using timed AI. Animals with very low AFC's may have lower pregnancy rates to AI.

Further work will be needed to determine if changing the duration of treatment with exogenous progesterone or introducing treatments that lower concentrations of progesterone before AI can be used to improved fertility in ovulatory animals receiving a timed AI. Dose response studies are needed in anovulatory animals to determine the optimum dose of progesterone before AI. Any potential impact of low AFC's on fertility requires validation and further investigation.

Table of Contents

1 Background

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 one of the key drivers of profitability in grazing enterprises in tropical environments (McGowan *et al.* 2014). It is dependent on multiple factors which affect the ability of cows to conceive and maintain pregnancies (Burns *et al.* 2010). Some of these factors include physiological events that occur before, during and after ovulation which orchestrate a series of finely tuned processes that play key roles in the establishment and maintenance of pregnancies (Walsh *et al.* 2011).

Concentrations of the hormone progesterone before and after ovulation are known to affect fertility by influencing the quality of oocytes and embryonic development. In several studies, higher concentrations of progesterone before AI have been demonstrated to enhance fertility (Fonseca *et al.* 1983; Folman *et al.* 1990; Echternkamp and Thallman 2011; Bisinotto *et al.* 2015a), improve embryo quality and reduce rates of early embryonic loss (Wiltbank et al., 2011). The potential mechanisms by which progesterone may influence fertility may vary depending on which physiological factors are affected. Concentrations of progesterone can affect the number of follicular waves, duration of dominance, growth rates and maximum diameters of preovulatory follicles which all may have an impact on fertility particularly if fixed-timed AI is used (Kinder et al., 1996; Townson et al., 2002; Carvalho et al., 2008). Progesterone also appears to play a key role in oocyte maturation and appears to have an antiapoptotic effect on oocytes and therefore could affect oocyte quality (Fair and Lonergan 2012). Low concentrations of progesterone before AI may also alter uterine function post AI by promoting premature uterine synthesis of PGF2α thus increasing the probability of luteolysis occurring following AI (Cerri et al. 2011). Other apparently unknown mechanisms also appear to exist (Fair and Lonergan 2012).

In *Bos indicus* cattle that have had their oestrous cycles synchronised for a timed AI lower concentrations of progesterone before a timed AI have been associated with an increase in the diameters of the largest follicle within ovaries at the end of treatment and at the time of AI in some studies (Carvalho *et al.* 2008; Martins *et al.* 2014). Fertility has, however, been variable with an increase in pregnancy rates being recorded in some studies (Dias *et al.* 2009; Meneghetti *et al.* 2009; Peres *et al.* 2009; Martins *et al.* 2014) but not in others (Phillips *et al.* 2004; Carvalho *et al.* 2008; Sá Filho *et al.* 2014). It also remains uncertain if responses to different circulating concentrations of progesterone before AI would differ between females that have, or have not ovulated before starting a treatment protocol that synchronises oestrus as the ovulatory status before commencing studies have not always been determined. Most AI strategies in studies that have investigated the potential effects of varying concentrations of progesterone before AI have also used a single fixed-time AI. Greater pregnancy rates to a timed AI have been found in animals that are detected in oestrus before AI compared to those that have not been detected in oestrus (Echternkamp and Thallman 2011). Use of a single, fixed-time AI strategy compared to AI on detection of oestrus can increase the likelihood that immature follicles may be induced to ovulate and decrease pregnancy rates at a synchronised oestrus or ovulation. Artificial insemination of some females on detection of oestrus at 48 hours after removing inserts and delaying forced AI to 72 hours after removing inserts in remaining animals may allow females with immature follicles more time for follicles to mature. This may help reduce any negative effects of

immaturity of ovarian follicles at the time of removing IVD's on fertility. Assessment of effects of different concentrations of progesterone before AI when using this modified AI strategy have not previously been investigated.

Evidence obtained from dairy cows suggests that the number of antral follicles ≥ 3 mm within the ovaries of dairy cows is positively associated with ovarian size (Ireland *et al.* 2008), fertility (Mossa *et al.* 2012), responses to superovulatory treatments (Singh *et al.* 2004; Ireland *et al.* 2007; Rico *et al.* 2009) and concentrations of progesterone following oestrus (Jimenez-Krassel *et al.* 2009). There have been no reports to date as to whether the number of antral follicles in *Bos indicus* heifers or cows is associated with fertility. Evidence of such a relationship could provide a basis for selection of more fertile animals in the future or for managing herds. The antral follicle count (AFC) of potential breeding animals can be optimised as factors such as prenatal maternal nutrition (Evans *et al.* 2012) and diet (Garnsworthy et al., 2009) can affect antral follicular counts in cattle.

The aim of this study was to determine the effects of altering concentrations of progesterone before AI on fertility in *Bos indicus* heifers and cows with synchronised oestrous cycles and to determine if AFC's had any association with fertility. Our hypotheses were that higher circulating concentrations of progesterone during the emergence of a potential preovulatory follicle will improve pregnancy rates to AI in anovulatory and ovulatory *Bos indicus* females when using an insemination strategy that involves AI on detection of oestrus at 48 hours after removal of IVD's and a timed insemination of remaining animals about 72 hours after removal of IVD's. Our second hypothesis was that there would be a positive association between AFC prior to a synchronised ovulation and fertility in *Bos indicus* females.

2 Projective objectives

The objectives of this project were:

1. To evaluate whether high physiological concentrations of progesterone during the emergence of preovulatory follicles and coincident with treatment with an intravaginal progesterone releasing device, improves pregnancy rates in *Bos indicus* cattle.
2. To indicate the merit of further studies aimed at manipulating concentrations of progesterone and increasing antral follicle number during preovulatory follicular development.

3 Methodology

3.1 Approvals

The experimental procedures were approved by the James Cook University Animal Ethics Committee (Approval number: A1856).

3.2 Location of animals

Three experiments were conducted across 3 years (2013 to 2015) using *Bos indicus* (Brahman) heifers and cows. In 2013 and 2015 animals were located on pasture at the James Cook University Tropical Veterinary Research Station, Fletcherview (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 (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. Average annual rainfall during this period of time was below annual median values with the pasture supply being estimated to be below that required to provide maintenance energy requirements in the weeks before commencing studies in 2013 and 2015. In 2014 cattle were relocated to the Swans Lagoon before and during the period of AI to ensure at least maintenance requirements were met. In 2015 persistence of drought conditions resulted in the necessity for 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.

3.3 Experiment 1

3.3.1 Treatments

Diagrammatic illustrations of treatment protocols used in Experiments 1 and 2 are shown in Figure 1. *Bos indicus* heifers (n = 115; Brahman) aged from 20 to 22 months of age and non-lactating cows (n= 67; Brahman) aged from 4 to 9 years were enrolled in this study over a 2-year period (2014 to 2015). All heifers and cows were initially weighed, condition scored (1 = emaciated, 9 = obese; (Wagner *et al.* 1988)) and had ovaries examined with transrectal ultrasonography (Eureka SA-600, Medison; 7.5 MHz probe) to determine if a CL was visible or not. At this time animals were administered 0.5 mg IM of cloprostenol (Estroplan®, Parnell Laboratories, Alexandria, NSW). Twelve days later (Day 0) animals without a detectable CL on 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. Animals were stratified by type (heifer or cow), and bodyweight and then randomly allocated to one of two treatments.

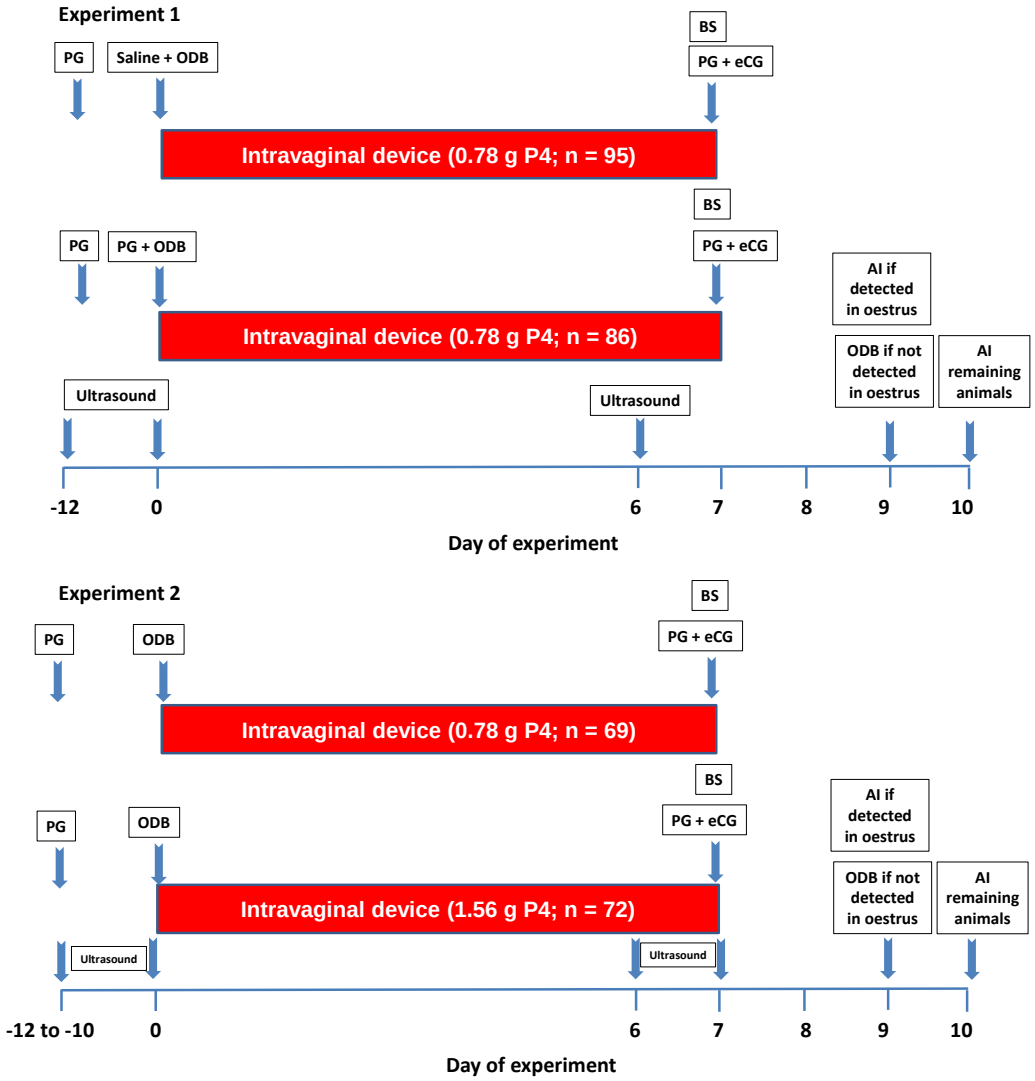

Figure 1. Outline of the treatment protocols used in Experiments 1 and 2. Animals were first examined for the presence of a CL between Days -12 and 10 and administered cloprostenol (PG; 0.5 mg IM). Animals without a CL detectable on the first examination were re-examined with ultrasound on Day 0. Animals with a CL were allocated to Experiment 1 in 2014 and 2015 while animals without a CL at both examinations were allocated to Experiment 2 in 2013 and 2015. Animals in Experiment 1 on Day 0 were administered an intravaginal device containing 0.78 g of progesterone (P4), oestradiol benzoate (ODB; 1 mg/500 kg) and then either saline (2 mL) or cloprostenol (0.5 mg) intramuscularly. Animals were subjected to transrectal ultrasonography on day 6. On Day 7 a blood sample (BS) was taken, IVD's were removed, cloprostenol (0.5 mg IM) and equine chorionic gonadotrophin (eCG; 400 IU IM) were administered and aids for the detection of oestrus applied. Animals detected in oestrus on Day 9 were artificially inseminated and the remaining animals were administered ODB (1 mg/500 kg IM) and inseminated 22 to 26 hours later. In Experiment 2 animals received similar treatments except an IVD containing either 0.78 or 1.56 g of progesterone was administered and ultrasound was performed on either Days -12 (2015) or -10 (2013) and Day 6 (2014 and 2015) or 7 (2013).

On Day 0 an intravaginal progesterone releasing device (IVD) containing 0.78 g of progesterone (Cue-Mate, Bioniche Animal Health, Armidale, NSW) was inserted into every animal and oestradiol benzoate (ODB; 1 mg/500 kg IM, Bomerol, Bayer Australia, Pty Ltd,

Pymble NSW) was administered. Animals were then either administered 2 mL of 0.9% saline solution IM (Saline) or 0.5 mg of cloprostenol IM (PG). Inserts were removed 7 days later (Day 7) and 0.5 mg of cloprostenol and 400 IU of eCG (Pregnecol, Bioniche Animal Health, Armidale, NSW) were administed IM and aids for the detection of oestrus (Estrotect, Genetics Australia, Bacchus Marsh, VIC) were applied. Forty eight hours after removal of inserts, animals that were detected in oestrus (>25% background colour of oestrous detection aid visible) were artificially inseminated with frozen and thawed semen while those that were not detected in oestrus were treated with ODB (1 mg/500 kg IM). After 22 to 26 hours later animals that had not yet been inseminated were inseminated. Bulls were placed with the herd 15 days later (Day 25) and removed after 12 weeks in 2014 and 10 weeks in 2015. The shorter breeding season in 2015 was introduced as part of a drought management plan in association with a reduction in stock numbers at the experimental site.

3.3.2 Ovarian ultrasonography and blood sampling

In 2015 a subset of 24 ovulatory heifers (n =13, Saline treatment and n = 11 PG treatment) were examined once daily from Days 2 to 7, then once daily until ovulation and then again on Day 14 using transrectal ultrasongraphic examination of the ovaries with a 7.5 MHz transducer (Mylab 5; Medical Plus Australia Pty Ltd, Tullamarine, Vic). Transrectal ultrasonography of the ovaries was also conducted on every animal in the study in 2014 and 2015 on Day 6 using the same ultrasound device. Video recordings of each ultrasound 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. Day of emergence was defined as the day on which the follicle that was classified as being dominant on Day 8 was first detected at a diameter ≥ 3 mm by transrectal ultrasonography. If emergence was determined to occur before Day 2 (n = 1) then the day of emergence was estimated using the mean growth rate between Day 2 and 8.

In heifers subjected to daily ultrasound examinations a blood sample was collected on Days 0, 2, 5, 7, 17 and 23. Every animal in the study was blood sampled on Day 7 and in 2014 every animal was also blood sampled on Day 25. Blood samples were collected from the coccygeal vein or artery into heparinized 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 isolated and stored at -20^{o}C until the time of assay.

3.3.3 Artificial insemination and pregnancy diagnosis

Artificial insemination was carried out using frozen-thawed semen by a single operator throughout the study. Bulls were introduced into herds 2 weeks after the completion of AI and removed after 10 weeks. Pregnancy diagnosis was performed on Day 61 (2014) and 56 (2015) with the aid of transrectal ultrasonography using a 7.5 mHz linear array transducer and again at least 5 weeks after removal of bulls.

3.4 Experiment 2

3.4.1 Treatments

As in Experiment 1, heifers and cows were weighed and examined initially on Day -10 (heifers in 2013) or Day -12 (cows in 2015) and had ovaries examined with transrectal ultrasonography (Eureka SA-600, Medison; 7.5 MHz probe) to determine if a CL was visible or not and cloprostenol (0.5 mg IM) was administered. Animals were re-examined on Day 0 and those in which a CL was not visible in an ovary at either examination were classified as anovulatory and an IVD containing 0.78 g or 1.56 g of progesterone (Cue-Mate) was inserted and oestradiol benzoate (1 mg/500 kg IM) was administered. After Day 0 treatments and AI were then identical to those applied within Experiment 1 with inserts being removed on Day 7.

3.4.2 Ovarian ultrasonography and blood sampling

Transrectal ultrasonography of the ovaries and constructing of follicle maps was conducted by the same operator and equipment used in Experiment 1. Examinations were conducted on every animal in 2013 on Day 7 and again at the time of AI. In 2015 ovaries were examined in all animals on Day 6. All animals were blood sampled and processed on Day 7 from the coccygeal vein or artery as per Experiment 1.

3.4.3 Artificial insemination and pregnancy diagnosis

Artificial insemination, natural mating with bulls and pregnancy diagnosis were carried out as per Experiment 1 with pregnancy diagnosis being carried out on Day 57 in 2013 and Day 56 in 2015 and at least 5 weeks after removal of bulls.

3.5 Experiment 3

3.5.1 Treatments

In 2014, 5 months before enrolling animals in Experiment 1 or 2, 40 anovulatory *Bos indicus* heifers (310 ± 2.7 kg) in which no CL was observed in the ovaries using transrectal ultrasonography between Days –13 and 0 were allocated to be treated with two IVD's (Cue-Mate, Bioniche Animal Health, Armidale, NSW). The total dose of progesterone administered with both devices was 0.78 g, 1.56 g, 2.34 g or 3.12 g of progesterone (10 animals per treatment). This was achieved by fitting 1, 2, 3 or 4 progesterone impregnated pods and 3, 2, 1 and 0 progesterone-free pods to the inserts, respectively with a total of two pods being fitted to each insert. Devices were inserted on Day 0, concurrently with administration of oestradiol benzoate (1 mg/500 kg IM). Inserts were removed on Day 7, aids for the detection of oestrus applied (Estrotect) and eCG (400 iu IM) was administered. Forty eight hours after removal of inserts, animals that were not detected in oestrus were treated with ODB (1 mg/500 kg IM) and were not inseminated.

3.5.2 Ovarian ultrasonography and blood sampling

Animals were subjected to transrectal ultrasonography as described for Study 1 on Days -13, 0, 2, 4, 6, 7, 9-12, 15, 20 and 25. Blood samples were collected on Days 0, 2, 4, 7, 15, and 20 for determination of concentrations of progesterone. Samples were collected and

processed as per Experiment 1. Ultrasound examinations were undertaken and ovarian maps were drawn and assessed by the same operator as in Experiments 1 and 2.

3.6 Hormone assays

Concentrations of progesterone in plasma in each experiment were determined using a radioimmunoassay 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 1.0, 5.9 and 9.3 ng/mL were respectively, 6.8% and 10.7%; 4.0 and 13.3%, and 6.5 and 10.7%. When concentrations of progesterone exceeded the standard curve they were diluted in charcoal stripped, foetal bovine serum (ThermoFisher, Subiaco, WA) before assay.

3.7 Statistical analyses

Statistical analyses were performed using the statistical software program IBM SPSS Statistics (version 20.0). Data were reported as mean ± SEM or in the case of AFC's as mean ± SD.

3.7.1 Experiment 1

In Experiment 1 analysis of variance (ANOVA) was used to compare the number and diameter of ovarian follicles on Day 6 between treatments. Year, age (heifer or cow) and treatment (all main interactions) were included as factors. Slopes (growth rates) and y-intercepts (estimates of potential day of follicle emergence) of emerging follicle growth curves were compared using the parallel line analysis function within SigmaPlot, (Ver. 12.5 Systat Software Inc.). Significant treatment by time interactions using repeated measures ANOVA confirmed that lines were not parallel.

Repeated measures analysis of variance was used to assess the effects of treatment on plasma concentrations of progesterone and the mean diameter of the emerging follicle between Days 4 and 8 that was classified as being dominant on Day 8. Follicles were classified as being dominant on Day 8 when the same follicle was subsequently detected to ovulate (sudden disappearance of an emerging follicle after Day 8 with the subsequent appearance of a CL in the same ovary) or if ovulation was not detected (n = 2) it was the largest follicle present on Day 8. The model included the main effects of treatment, time, age (heifer or cow) and the interaction of treatment and time, treatment and age and treatment, time and age. If Mauchly's test indicated violation of the assumption of sphericity, probability values were obtained after degrees of freedom were adjusted using the Greenhouse–Geisser statistic. Concentrations of progesterone on Day 25 in 2014 were compared using ANOVA. Treatment, age, whether animals were diagnosed pregnant to AI, the day of insemination and relevant interaction terms was always included in the model. Body weight, the diameter of the largest follicle recorded on Day 6 were included initially as covariates in the model but subsequently excluded when effects were found to be not significant. Where significant differences were detected means were compared using an independent sample t-test or a paired sample t-test .

Multivariable logistic regression was used to model the effect of treatment or plasma concentration of progesterone on Day 7 on outcome variables (pregnancy rate to AI, submission rate on Day 9 and final pregnancy rate). Variables such as body weight, body

condition score, 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. To avoid repetition of highly correlated variables treatment and concentrations of progesterone on Day 7 were used as explanatory variables in separate models as the variables were not independent. Terms were considered for elimination from each model using backwards step-wise logistic regression although treatment group, concentrations of progesterone on Day 7 and the AFC were retained in their respective models. The test for elimination was a likelihood-ratio test using a significance level of P ≥ 0.10. If an interaction was significant at *P* < 0.10, the associated main effects were included in the model. 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.

3.7.2 Experiment 2

Concentrations of progesterone at the time of removal of inserts was compared using ANOVA. Treatment and year were included as factors in the model. Analysis of variance was used to compare the diameters of the largest follicle on the ovary at the time of AI in heifers in 2013 and the number and diameter of ovarian follicles on Days 6 or 7 between treatments in 2013 and 2015. Age (heifer in 2013 or cow in 2015) and the interaction of treatment and age, were included as factors within the model. Effects of year and age were confounded so age was used to describe comparisons between years with the understanding that any differences detected could have been attributed to either age or year or both. Logistic regression analysis as described for Experiment 1 was used for examining potential association with the same binomial outcome variables as described for Experiment 1.

3.7.3 Experiment 3

Repeated measures analysis was used to compare the concentrations of progesterone between Days 0 and 20 and between the diameter of follicles that were classified as emerging on Day 6 between Days 6 and 10. The model included effects due to treatment, Day and whether heifers ovulated or not and relevant interactions. Multiple comparisons between treatments were compared using Tukey's test.

Weight was included as a covariate initially within ANOVA models but removed if found to be not significant. Proportional responses were compared using Pearson's chi-square test or Fisher's exact test when one or more of the cells had a frequency <10. *P*-values <0.05 were considered significant and 0.05 < *P* < 0.10 were regarded as a tendency.

4 Results

4.1 Experiment 1

4.1.1 Data exclusions

A total of 4 animals were excluded from all analyses due to incomplete data. This included one heifer in the group of 24 that was scanned daily. A total of 15 animals (6 in 2014 and 9 in 2015) were not available at the time when the final pregnancy test was undertaken and so were excluded from analysis of the final pregnancy rates. Two additional animals in the PG

treated group were excluded from analyses of follicle growth patterns as luteolysis was not induced in one animal and in the other animal a dominant follicle persisted in the ovary from Day 2 and was >11 mm in diameter between Days 3 and 8 and eventually ovulated. Data on AFC's were missing from one cow in Experiment 1. This cow was excluded from analyses of AFC's but included in other analyses.

4.1.2 Concentrations of progesterone

Circulating concentrations of progesterone in the subset of heifers repeatedly sampled between Days 0 and 23 are shown in Figure 2. Mean concentrations of progesterone varied over time ($P < 0.001$), between treatments ($P = 0.032$) and an interaction between treatment and time was detected ($P = 0.011$). Mean concentrations of progesterone were similar on Day 0 but on Days 2 to 7 they were less in heifers treated with PG compared with those treated with saline ($P < 0.05$). On Day 17 there was a tendency for concentrations of progesterone to be less in heifers treated with saline compared with those treated with PG (3.7 ± 0.44 ng/mL versus 5.1 ± 0.58 ng/mL; $P = 0.052$) but on Day 23 concentrations of progesterone were similar between treatments. To check for potential effects of the day of AI and pregnancy status to AI on concentrations of progesterone on Day 17 a separate ANOVA model was tested that included both of these factors and treatment in the same model. Both the day of AI and pregnancy status were not found to significantly affect concentrations of progesterone on Day 17 with this model ($P > 0.45$) and there was tendency for treatment to effect the concentrations of progesterone on Day 17 ($P = 0.077$).

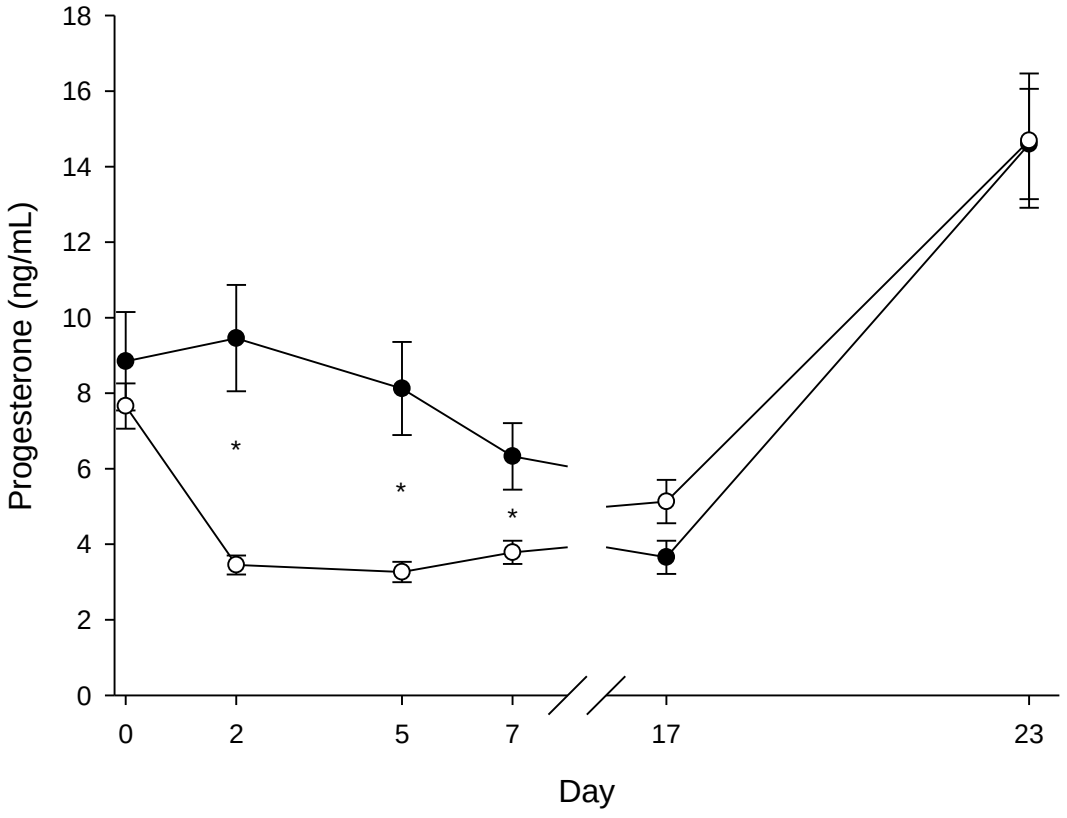

Figure 2. Mean ± SEM concentrations of progesterone in plasma in heifers during (Day 0 to 7) and after the period of insertion (Days 17 and 23) of intravaginal devices of a subset of heifers treated with

either saline (●) or cloprostenol (○) on Day 0, Mean concentrations of progesterone differed over time (P = 0.032) between treatments (P = 0.005) and there was an interaction between treatment and time (P = 0.011). * Indicates where means differed (P < 0.05).

In all of the animals in Experiment 1 mean concentrations of progesterone on the day of removal of inserts (Day 7) were greater in animals treated with saline compared to those treated with PG (P < 0.001) although mean differences between treatments were greater in 2015 compared to 2014 (Figure 3; treatment by year interaction; P = 0.043). Mean concentrations of progesterone did not vary between heifers and cows (P = 0.119) and no other significant interactions were found (P > 0.10).

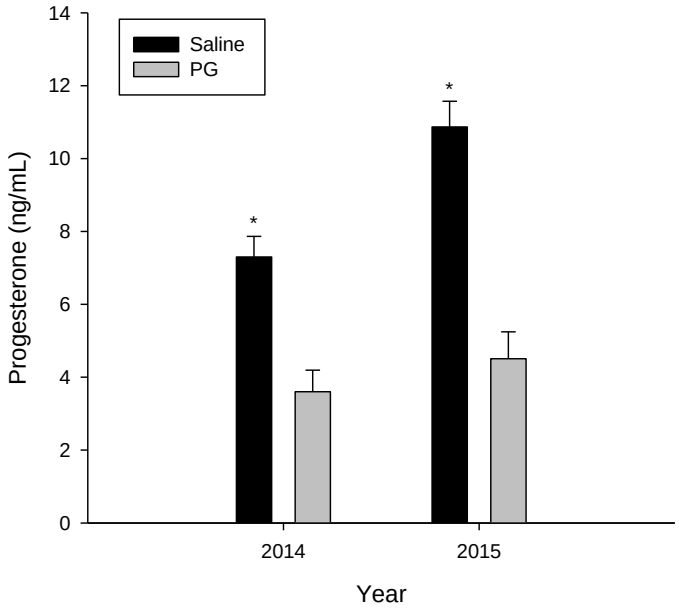

Figure 3. Concentrations of progesterone in plasma at the time of removal of intravaginal inserts in animals treated in each year in Experiment 1. Mean concentrations of progesterone differed within years (*; P < 0.001), between years (P = 0.001) and a treatment by year effect was detected (P = 0.043).

In 2014, on Day 25 mean concentrations of progesterone did not differ significantly between treatments (P = 0.553) but were greater in animals that were pregnant to AI compared to those that were not (14.6 ± 0.83 ng/mL verses 9.5 ± 0.80 ng/mL, respectively; P < 0.001) and tended to be greater in cows compared to heifers (12.8 ± 0.83 ng/mL compared to 10.9 ± 0.94 ng/mL, respectively P = 0.053). None of the interaction terms were significant (P > 0.290).

4.1.3 Ovarian follicular dynamics in heifers

The diameter of the largest follicle imaged within the ovary of all animals on Day 6 was less in animals treated with saline compared with those treated with PG (Table 1, 7.9 mm ± 0.21 versus 10.8 mm ± 0.31; P <0.001), was less in heifers compared to cows (8.9 mm ± 0.24 versus 10.1 mm ± 0.40; P = 0.008) and did not differ significantly between years (P = 0.339). Interaction terms included in the model were not significant (P > 0.10).

In a subset of heifers examined with ultrasound the diameter of the ovarian follicle that was determined to be dominant on Day 8, between Days 4 and 8 was affected by treatment (*P* = 0.002) and day (*P* <0.001) but a significant treatment by day interaction was not detected (*P* = 0.183). During this period of time the mean diameter of the emerging follicle was always smaller in heifers treated with saline compared to those treated with PG (*P* <0.05; Figure 4). The slope of each line was similar between treatments (*P* = 0.143) but the estimated y-intercepts differed (*P* <0.001). The day of emergence of the follicle that was classified as dominant on Day 8 occurred later in heifers treated with saline compared with those that were treated with PG (3.8 ± 0.33 versus 2.4 ± 0.42, *P* = 0.016).

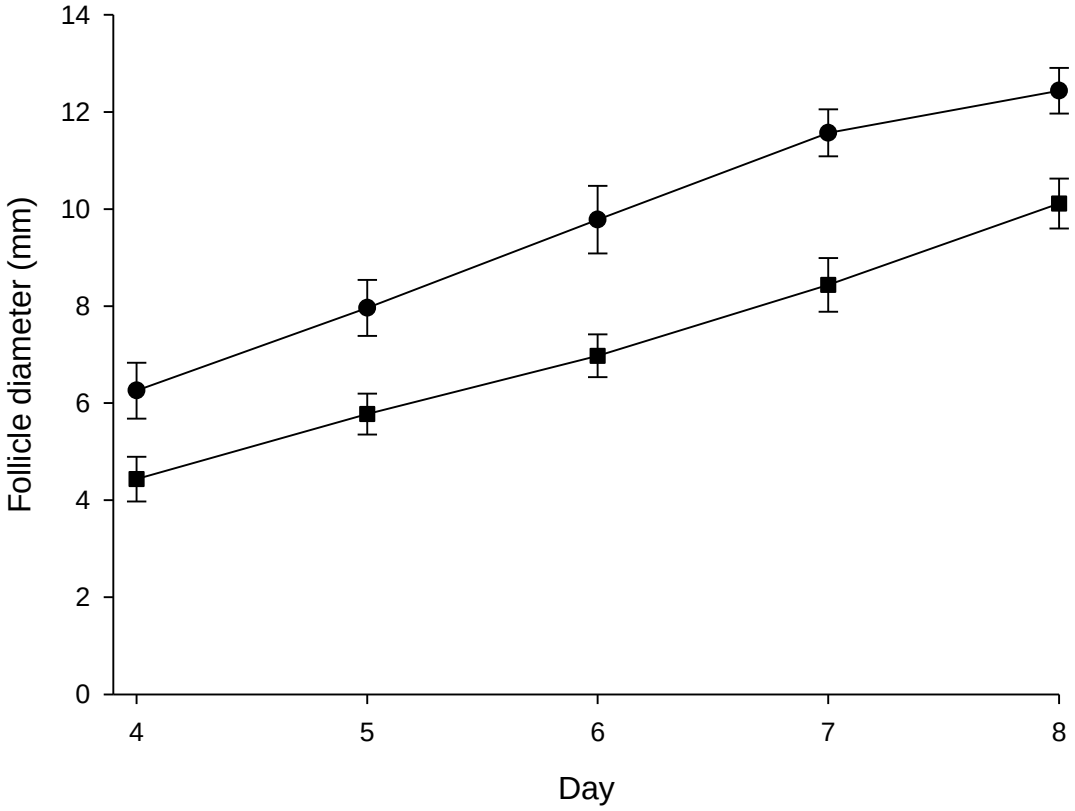

Figure 4. Mean ± SEM diameter of the follicle classified as dominant on Day 8 in heifers treated with an intravaginal progesterone releasing insert from Days 0 to 7, oestradiol benzoate and either saline (■) or cloprostenol (●) on Day 0 and eCG and cloprostenol on Day 7. Effect of treatment (*P* = 0.001), day (*P*< 0.001) and treatment by day (*P* = 0.183).

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 versus 50.0%, 4/8, for Saline and PG, respectively; *P* = 0.156). Pregnancy rates of those that were inseminated and pregnancy tested that ovulated before and after AI did not differ (40.0%, 2/5 versus 60.0%, 9/15, respectively; *P* = 0.617).

4.1.4 Submission rates to AI Day 9

The percentage of animals submitted to AI on Day 9 was affected by treatment with more animals treated with PG being submitted to AI on Day 9 compared to animals treated with

saline (74.1%, 63/85 versus 18.5%, 17/92 respectively; *P*<0.001; Tables 1 and 2;). There was evidence for a treatment by year interaction (*P* = 0.055; Table 2) with a greater percentage of animals submitted for AI on Day 9 in the Saline treated animals in 2014 compared to 2015 (28.6%, 14/49 versus 7.0%, 3/43, respectively). The odds of animals being submitted for AI on Day 9 was greater when the largest follicle in the ovary was ≥ 9 mm in diameter compared with animals in which the largest follicle in the ovary was <9 mm in diameter (Table 2). Other variables and interaction terms examined did not significantly affect submission rates (*P* >0.10). Using a second model where the concentration of progesterone on Day 7 was included as an explanatory variable in the model instead of treatment, the concentration of progesterone on Day 7 affected submission rates on Day 9 (*P* < 0.001; Table 2) with animals with lower concentrations of progesterone on Day 7 having a higher odds of being submitted for AI on Day 9. Using this model animals in which the largest follicle imaged in the ovary was ≥ 9 mm, again had greater odds of being submitted for AI on Day 9 than those with follicles < 9 mm in diameter. Other variables and interaction terms were not significant (*P* > 0.10). The probability of animals being submitted for AI in relation to the concentration of progesterone on Day 7 is shown in Figure 5.

4.1.5 Pregnancy rates

Pregnancy rates to AI were not significantly affected by treatment with saline or PG (45.7%, 42/92 versus 51.8%, 44/85, respectively; *P* = 0.411; Table 1 and 2). The AFC on Day 6 (*P* = 0.375; Table 2), along with age, bull, weight, the diameter of the largest follicle on Day 6 or any relevant interaction terms did not significantly affect pregnancy rates (*P* > 0.10). In a separate model that included concentration of progesterone on Day 7, an interaction between concentrations of progesterone on Day 7 and year was detected (*P* = 0.022; Table 2) with lower concentrations of progesterone on Day 7 increasing the odds of pregnancy to AI in 2014 (*P* = 0.017) but not in 2015 (*P* = 0.441). The AFC (*P* = 0.389) and other main effects and interaction terms, did not significantly affect pregnancy rates using this model (*P* >0.10; Table 2). The probability of pregnancy for animals with differing concentrations of P4 on Day 7 in 2014 is illustrated in Figure 6.

Table 1. Details of the number of animals assigned to different treatments in Experiments 1 and 2, diameter of the largest follicle imaged in the ovary on Day 6 or 7, submission rates on Day 9 and pregnancy rates.

Variable	Year	Experiment – Cycling status			
		1 - Ovulatory		2 - Anovulatory	
Treatment		Saline	PG	0.78 g of progesterone	1.56 g of progesterone
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 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 to AI (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]

Table 2. Multivariable logistic regression analysis of submission rates on Day 9 and pregnancy rates in Experiment 1.

Dependent variable	Explanatory variable	df*	*P*	Odds ratio (95% CI*)	Reference group
Submitted for AI Day 9 (Model 1)	Saline treatment	1	<0.001	0.04 (0.01-0.16)	PG
	Year 1	1	0.860	0.91 (0.32-2.6)	Year 2
	Follicle <9 mm Day 6	1	<0.001	0.20 (0.09-0.43)	Follicle ≥ 9 mm
	Treatment x Year	1	0.055	5.1 (0.91-28.3)	-
Submitted for AI Day 9 (Model 2)	Progesterone	1	<0.001	0.65 (0.55-0.77)	-
	Follicle <9 mm Day 6	1	<0.001	0.17 (0.07-0.37)	Follicle ≥ 9 mm
Pregnancy rate to AI (Model 1)	Saline treatment	1	0.399	0.77 (0.43-1.4)	PG
	Antral follicle count	1	0.375	1.0 (0.95-1.1)	-
Pregnancy rate to AI (Model 2)	Progesterone	1	0.501	1.0 (0.94-1.1)	-
	Antral follicle count	1	0.389	1.0 (0.93-1.1)	-
	Year 1	1	0.152	2.30 (0.78-6.8)	Year 2
	Progesterone x Year (2014)	1	0.022	0.85 (0.74-0.98)	2015
Final pregnancy rate	Saline treatment	1	0.171	1.87 (0.75-4.7)	PG
	Follicles ≥ 3 mm	1	0.216	0.92 (0.80-1.0)	-
	Year	1	0.062	2.33 (0.94-5.8)	Year 2

*df, degrees of freedom; CI, confidence interval.

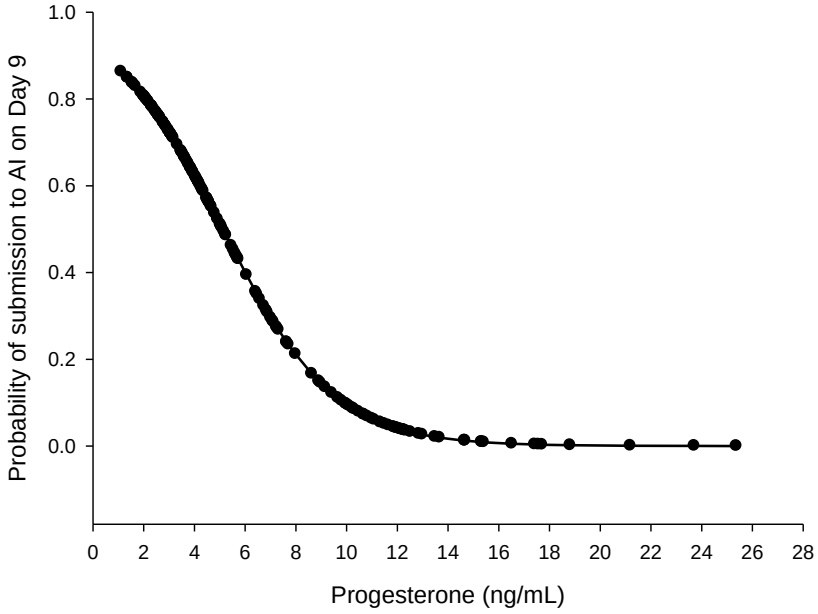

Figure 5. A fitted curve of the relationship between the concentration of progesterone (ng/mL) at the time of removing intravaginal devices (Day 7) and the probability of submission to AI on Day 9 (*P* < 0.001)

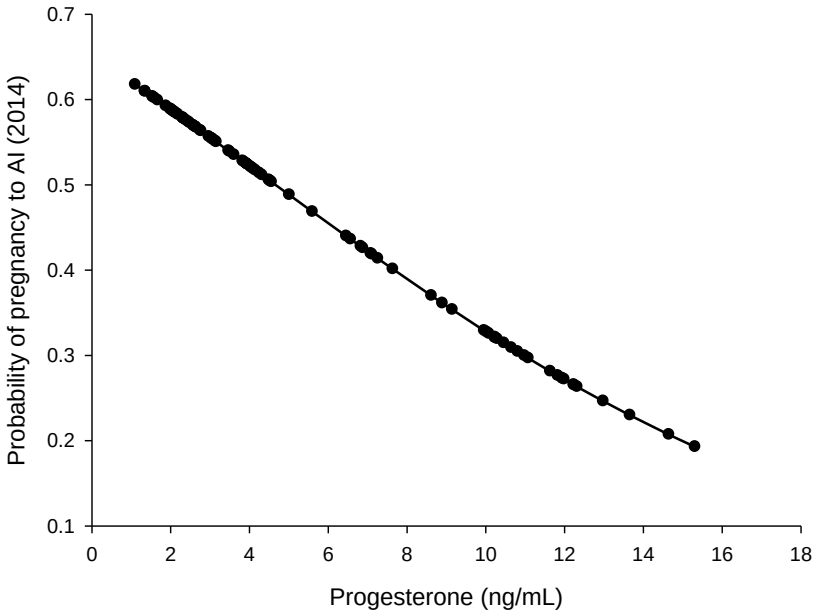

Figure 6. A fitted curve of the relationship between the concentration of progesterone (ng/mL) at the time of removing intravaginal devices (Day 7) and the probability of pregnancy to AI in Experiment 1 in 2014 (*P* = 0.022).

Final pregnancy rates at the end of the breeding season were not affected by treatment (*P* = 0.171) or the AFC (*P* = 0.216) but tended to be greater in 2014 compared to in 2015 (89.8%, 79/88 versus 79.7% 59/74, respectively; *P* = 0.062).

4.2 Experiment 2

4.2.1 Data exclusions

Two cows (one in each treatment group) were eliminated from the study due to missing data.

4.2.2 Concentrations of progesterone

Concentrations of progesterone on Day 7 were less in animals treated with a lower compared with a higher dose of progesterone (3.39 ± 0.20 ng/mL versus 4.27 ± 0.16 ng/mL, respectively; *P* = 0.001). Mean concentrations of progesterone were greater in heifers in 2013 compared to cows in 2015 (4.20 ± 0.27 ng/mL versus 3.63 ± 0.14 ng/mL, respectively; *P* = 0.040). A treatment by age (heifer or cow) interaction was not detected (*P* = 0.626).

4.2.3 Ovarian follicles

The diameter of the largest follicle in the ovary on Day 6 or 7 was greater in animals treated with a lower compared to a higher dose of progesterone (9.4 ± 0.27 mm versus 8.8 ± 0.25 mm; *P* = 0.039) and was greater in heifers in 2013 compared to cows in 2015 (9.7 ± 0.28 mm versus 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 a higher dose of progesterone (13.0 ± 0.63 mm versus 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 progesterone ovulated before the time of AI (41.7%, 10/24 versus 14.8%, 4/27, respectively; *P* = 0.032).Pregnancy rates to AI were lower in those heifers that had ovulated compared to those that had not ovulated by the time of AI (42.9%, 6/14 versus 75.7%, 28/37, respectively; *P* = 0.027).

4.2.4 Submission rates to AI Day 9

A treatment by age interaction (*P* = 0.033) was found for the percentage of animals submitted for AI 2 days after removal of inserts (Table 3). A greater percentage of cows in 2015 that were treated with a lower dose of progesterone were submitted to AI 48 hours after removing inserts compared to those treated with a higher dose of progesterone (33.3%, 15/45 versus 13.3%, 6/45, respectively; *P* = 0.025; Table 2). In 2013 the same comparison in heifers failed to reveal a significant differences in the percentages submitted to AI 48 hours after removing inserts (12.5%, 3/24 versus 25.9%, 7/27, low versus high dose of progesterone, respectively; *P* = 0.228). Submission rate at 48 hours after removal of inserts also differed with the diameter of the largest follicle on Day 6 or 7 (*P* < 0.001; Table 3), with the submission rate being less in animals in which the largest follicle was < 9 mm in diameter compared to those with follicles ≥ 9 mm diameter (7.7%, 5/65 versus 34.2%, 26/76, respectively). In a separate model that included concentration of progesterone at the time of removal of inserts but not treatment, the concentration of progesterone was not significantly associated with submission rate 2 days after removal of inserts (*P* = 0.350) but again whether follicles were greater or lesser than 9 mm in diameter (*P* <0.001) was associated with submission rate (data not shown).

Table 3. Multivariable logistic regression analysis of submission rates on Day 9 and pregnancy rates in Experiment 2.

Dependent variable	Explanatory variable*	*P*	Odds ratio (95% CI*)	Reference group
Submitted for AI Day 9 (Model 1)	Low P4	0.098	2.5 (0.82-7.7)	High P4
	Heifer*	0.333	1.9 (0.52-6.8)	Cow
	Follicle <9 mm Day 6	<0.001	0.17 (0.06-0.47)	Follicle ≥ 9 mm
	Treatment x age	0.033	0.14 (0.02-0.91)	
Pregnancy rate to AI (Model 1)	Low P4	0.146	0.58 (0.28-1.2)	High P4
	Antral follicle count	0.331	1.1 (0.95-1.2)	
	Heifer†	<0.001	3.80 (1.8-8.0)	Cow
	Follicle <9 mm Day 6 or 7	0.050	0.48 (0.23-1.01)	Follicle ≥ 9 mm
Pregnancy rate to AI (Model 2)	Progesterone at removal	0.045	1.3 (0.99-1.7)	-
	Antral follicle count	0.386	1.1 (0.94-1.2)	-
	Heifer†	<0.001	3.5 (1.6-7.4)	Cow
	Follicle <9 mm Day 6 or 7	0.060	0.50 (0.24-1.0)	Follicle ≥ 9 mm
Final pregnancy rate	Low P4	0.823	0.88 (0.29-2.7)	High P4
	Antral follicle count	0.161	1.1 (0.95-1.4)	
	Heifer†	<0.001	8.5 (2.7-26.8)	Cow

*degrees of freedom, df=1 for all variables; CI, confidence interval.

†Animal type (heifer or cow) was confounded by year so variations between heifers and cows could also be due to variations between years, animal types or both.

4.2.5 Pregnancy rates

Pregnancy rates were not affected by treatment (P = 0.146) or AFC (*P* = 0.386) but the odds of pregnancy were greater in heifers compared with cows (*P* <0.001) when the largest follicle on the ovary on Day 6 or 7 was ≥ 9 mm in diameter (*P* = 0.050; Table 3). In a second model that included the concentration of progesterone at the time of removal of inserts the odds of pregnancy were greater in animals with greater concentrations of progesterone at the time of removal of inserts (*P* = 0.045), in heifers compared to cows (*P* < 0.001) and tended to be greater in animals with larger follicles on Day 6 (*P*= 0.060; Table 3). The odds of pregnancy were not significantly associated with AFC (*P* = 0.386). Final pregnancy rates at the end of the breeding season were not affected by treatment (*P* = 0.823) or the AFC (*P* = 0.161) but were greater in heifers compared to cows (80.4%, 41/51 versus 30.4% 7/23, respectively; Table 3; *P* < 0.001).

4.3 Experiment 3

4.3.1 Data exclusions

One heifer treated with 1.56 g of progesterone was eliminated from the study as ovulation occurred between Day 0 and 2 and a CL was observed to subsequently develop during treatment with an IVD.

4.3.2 Concentrations of progesterone

Concentrations of progesterone in treated heifers between Days 0 to 20 of the experiment are shown in Figure 7. Significant effects of treatment (P <0.001), Day (P <0.001), whether heifers ovulated or not (P = 0.002) and significant interactions between treatment and time (P < 0.001), and whether heifers ovulated or not and time (P = 0.050) were detected. No significant interaction between treatment and whether heifers ovulated or not was detected (P = 0.413). Mean concentrations of progesterone were similar between treatments on Days 0, 15 and 20 (P > 0.05). On Day 2 concentrations were significantly greater in the heifers treated with 2.34 g and 3.12 g of progesterone compared to those treated with 0.76 g and 1.56 g of progesterone. On Day 4 mean concentrations of progesterone were significantly less in heifers treated with 0.76 g of progesterone compared with the other treatments and less in heifers treated with 1.56 g of progesterone compared with those treated with higher doses of progesterone. On Day 7 mean concentrations of progesterone were significantly less in heifers treated with 0.76 g and 1.56 g of progesterone compared to those treated with higher doses. Heifers that ovulated had greater concentrations of progesterone on Days 15 and 20 than heifers that did not ovulate (data not shown; P < 0.05).

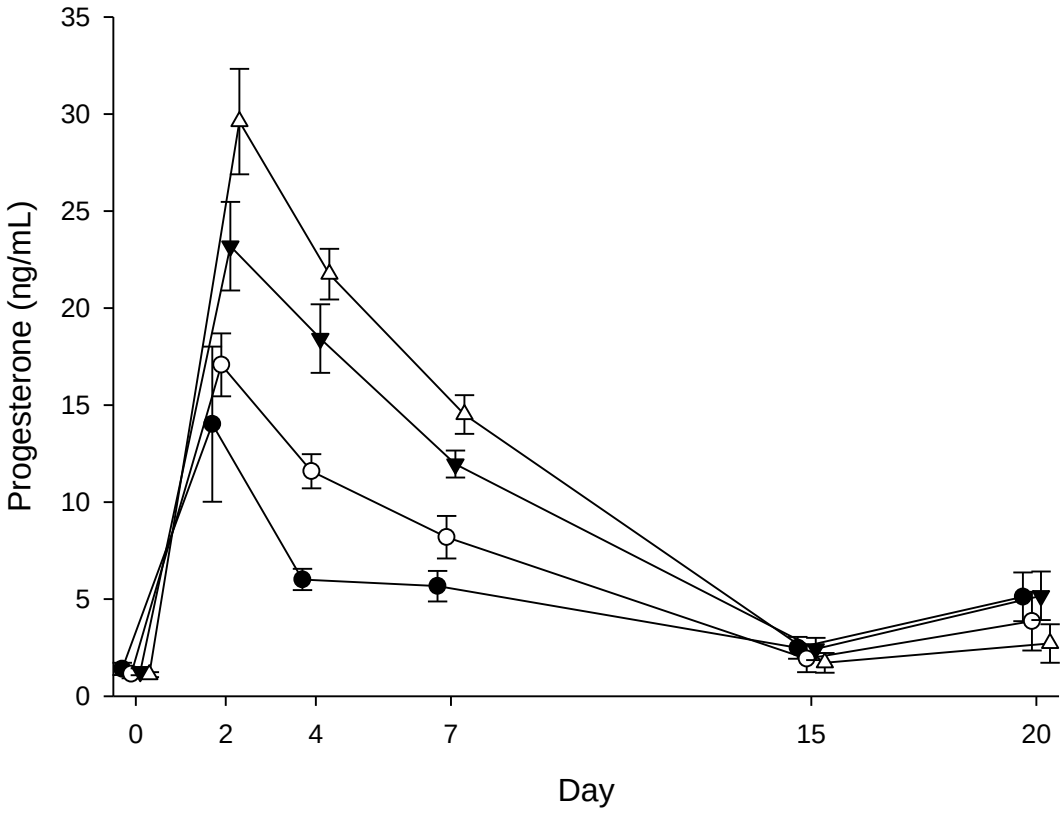

Figure 7. Mean ± SEM concentrations of progesterone in plasma in heifers treated between Days 0 and 7 with intravaginal progesterone releasing devices containing 0.76 g (●), 1.56 g(○) 2.34 g (▼) and 3.12 g (△) of progesterone.

4.3.3 Ovarian follicular dynamics

Between Days 6 and 10 the diameter of the largest follicle that was retrospectively determined to be emerging on Day 6, was found to differ between treatments (P = 0.022; Figure 8a) and was greater in heifers that ovulated compared to those that remained anovulatory (Figure 8b; P < 0.001). A significant interaction between day and whether heifers ovulated or not was also detected (P < 0.001; Figure 8b), but a treatment by day interaction was not detected (P = 0.415). The slopes of the lines of follicle growth in heifers treated with four doses of progesterone did not differ significantly (Figure 8b; P = 0.206) but the estimated y-intercepts did (P < 0.001). The slope of the line (growth rates) of follicle diameter between Days 6 and 10 in the ovulatory heifers was greater than in the anovulatory heifers (Figure 8 b; P = 0.021). The mean diameter of emerging follicles between Days 6 and 10 was greater in heifers treated with 0.78 g compared to those treated with 1.56 g of progesterone (7.5 ± 0.41 mm versus 6.2 ± 0.40 mm, respectively; P < 0.05). Differences between other treatments were not significant. The mean difference between the diameter of the largest follicle in emerging follicles that did and did not ovulate increased over time and was greater on Days 9 and 10 compared to Days 6 and 7 (Figure 3b). The mean diameter of the ovulatory follicle (10.3 ± 0.21 mm; P = 0.546) or the diameter of the largest follicle imaged within ovaries on both Days 7 and 9 (P > 0.80) and the percentage of heifers ovulating in each treatment group (56.4%, 22/39; P = 0.227) did not differ significantly between treatments.

The percentage of heifers detected in oestrus within 120 h of removing inserts did not differ between treatments (69.2%, 27/39; P = 0.870). The percentage of ovulatory compared to anovulatory heifers that were detected in oestrus during this time were similar (77.3%, 17/22 versus 58.8%, 10/17; P = 0.216). A positive association between follicle diameter on Day 7 and the probability of ovulation was found (P < 0.001). Heifers with follicles greater than 9 mm in diameter on Day 7 had > 90% probability of ovulating.

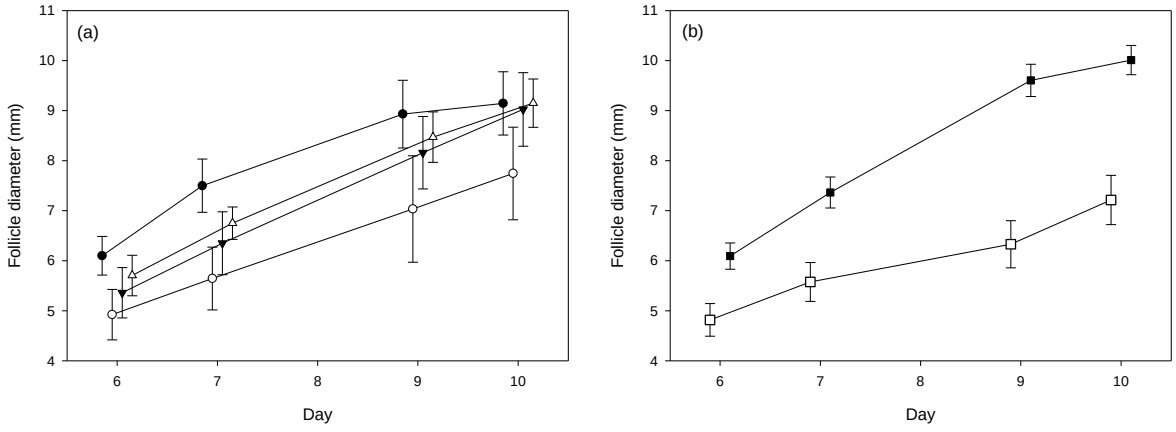

Figure 8. The diameter of the largest follicle of a cohort that was retrospectively determined to be emerging on Day 6, (a) for heifers treated with 0.78 g (●), 1.56 g(○) 2.34 g (▼) and 3.12 g (△) of progesterone, and, (b) for heifers that ovulated (■) or did not ovulate (□).

4.4 Combined data – Experiments 1 and 2

4.4.1 Submission rate Day 9

In the combined data set the odds that animals being submitted for AI 2 days after removal of inserts was greater in ovulatory compared to anovulatory animals (46.2%, 84/182 versus 21.4%, 30/140, respectively; *P* <0.001), in animals with lower concentrations of progesterone at the time of removing inserts (*P* <0.001) and in animals with follicles that were ≥ 9 mm in diameter on Day 6 or 7 (15.6%, 24/154 versus 53.3%, 89/167; *P* <0.001; Table 4). When potential associations between factors that might affect the odds of having a follicle ≥ 9 mm in diameter on Day 6 or 7 were examined only concentrations of progesterone at the time of insert removal was found to affect the dependent variable (*P* <0.001) with lower concentrations of progesterone increasing the odds that a follicle would be ≥ 9 mm in diameter on Day 6 or 7.

4.4.2 Pregnancy rates

The odds of animals being diagnosed as pregnant to AI were greater in ovulatory compared to anovulatory animals (48.6%, 86/177 versus 45.4%, 64/141, respectively; model *P* = 0.008) and were greater in 2013 and 2014 compared to 2015 (*P* = 0.007). While concentrations of progesterone at the time of removal of inserts were not significantly associated with pregnancy rates, significant interactions were detected between the concentration of progesterone at insert removal and whether animals were ovulatory or not at the start of treatment (*P* = 0.024) and with year (*P*= 0.019; Table 4). Concentrations of progesterone at the time of removal of inserts were positively associated with pregnancy in the anovulatory but not the ovulatory animals and in 2014 lower concentrations at the time of removal were associated with significantly greater odds of pregnancy. Antral follicle count was not significantly associated with pregnancy rates to AI when entered as a continuous variable (Table 4) but if entered as a categorical variable with females classified as having AFC's ≤4 or >4 there was a tendency for pregnancy rates to AI to be less in females with AFC's ≤4 compared to those with AFC's >4 (31.4%, 16/51 versus 50.4%, 134/266, respectively; model *P* = 0.081).

Final pregnancy rates were not significantly affected by concentrations of progesterone at the time of insert removal but an interaction between concentration of progesterone and year was detected (*P* = 0.031; Table 4). In 2014 final pregnancy rates were positively associated with lower concentrations of progesterone at the time of removal of inserts. Final pregnancy rates were also lower in anovulatory compared to ovulatory animals (64.9%; 48/74 versus 85.2%, 138/162, respectively; *P* < 0.001) and were greater in 2014 compared to 2015 (80.4%, 41/51; 89.8%, 79/88; 68.0%, 66/97; for years 2013 to 2015, respectively; *P* = 0.031, Table 4). No significant association between AFC's and final pregnancy rates were found when AFC was entered either as a continuous (*P* = 0.975) or categorical variable as described for pregnancy rates to AI (71.9%, 23/32 versus 79.9%, 163/204 for females with AFC's of ≤ 4 and >4, respectively; *P* = 0.929). Mean (± SD) AFC was 7.6 ± 3.2 (range: 1 to 20; Figure 9).

Table 4. Multivariable logistic regression analysis of the combined data from Experiments 1 and 2 for variables found to significantly affect submission rates on Day 9, having a follicle ≥ 9 mm in diameter on Day 6 or 7 and pregnancy rates to AI.

Dependent variable	Explanatory variable	df*	*P*	Odds ratio (95% CI*)	Reference group
Submission rate Day 9	Anovulatory	1	<0.001	0.13 (0.07-0.24)	Ovulatory
	Follicle <9 mm Day 6	1	<0.001	0.17 (0.09-0.31)	Follicle ≥9 mm
	Progesterone	1	<0.001	0.68 (0.60-0.79)	-
Follicle ≥ 9 mm in diameter Day 6 or 7	Progesterone	1	<0.001	0.85 (0.79–0.91)	-
Pregnancy rate to AI	Progesterone at removal	1	0.559	1.02 (0.94-1.1)	-
	Anovulatory	1	0.008	0.11 (0.02-0.61)	Ovulatory
	Antral follicle count	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
	Progesterone at removal x cycling status	1	0.024	1.5 (1.0–2.2)	-
	Progesterone at removal x year	2	0.019	-	-
	Progesterone x year (2013)	1	-	0.66 (0.41-1.1)	2015
	Progesterone x year (2014)	1	-	0.85 (0.74–0.98)	2015
Final pregnancy rate	Progesterone at removal	1	0.335	1.1 (0.94-1.2)	-
	Anovulatory	1	<0.001	0.14 (0.04-0.42)	Ovulatory
	Antral follicle count	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
	Progesterone at removal x year	2	0.031		
	Progesterone x year (2013)	1		0.75 (0.53-1.1)	2015
	Progesterone x year (2014)	1		0.78 (0.63-0.97)	2015

*df, degrees of freedom; CI, confidence interval.

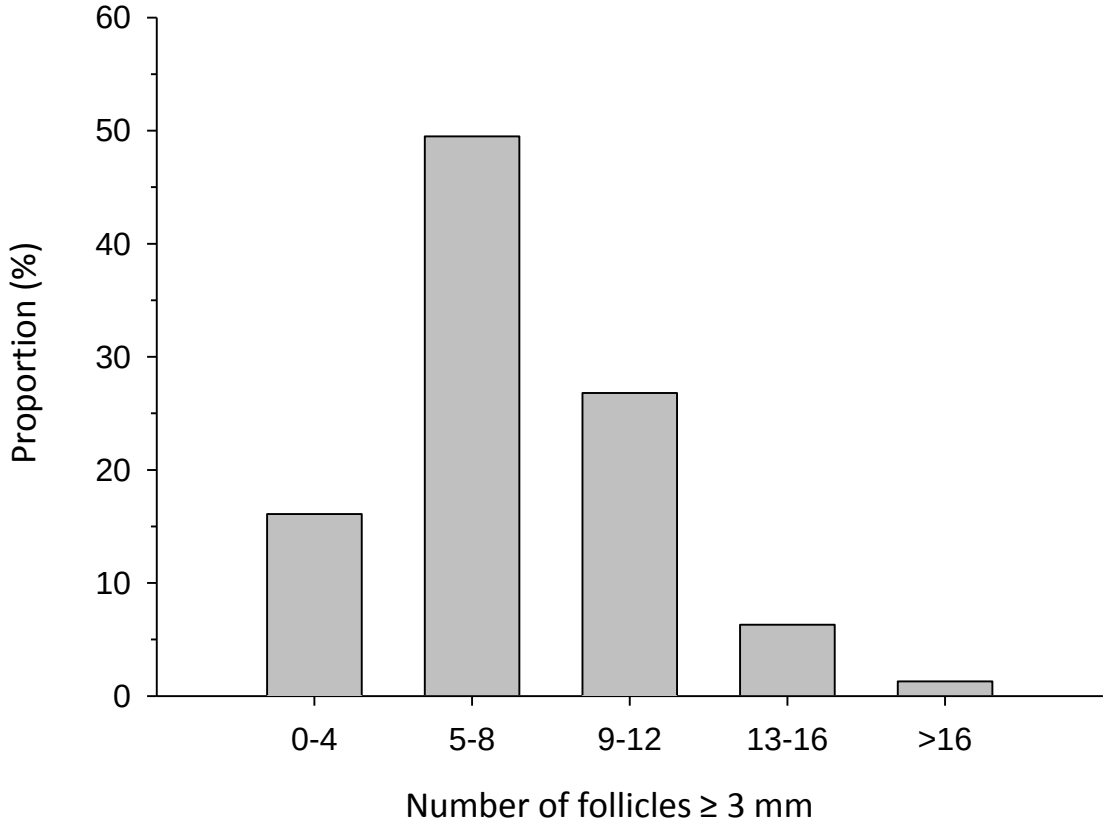

Figure 9. Frequency distribution of the total number of ovarian follicles ≥ 3 mm in diameter (AFC) in *Bos indicus* heifers and cows enrolled in Experiments 1 and 2 with ultrasound images recorded on Day 6 or 7 following administration of oestradiol benzoate and an intravaginal device containing progesterone.

5 Discussion

Optimising fertility when synchronising oestrus to AI is dependent on animal, environmental and physiological factors (Sa Filho *et al.* 2010). The results of this study highlight that responses to the synchronisation protocols applied in this study can be variable across years, treatments, cyclic status and ages. This highlights the complexity associated with optimising pregnancy rates to treatments across herds and how small variations in treatment protocols can yield differences in responses. Variability in pregnancy rates to AI in *Bos indicus* females is one of the main factors that limit the application of this technology for beef cattle within tropical environments. The results of this study highlight that increasing the percentage of animals cycling before starting treatments, increasing concentrations of progesterone in anovulatory animals, decreasing concentrations of progesterone in ovulatory animals and possibly eliminating animals with very low AFC's may be some factors that should be considered when trying to reduce variability in responses to treatments.

5.1 Follicle dynamics

Lower circulating concentrations of progesterone at the end of treatment with an IVD in this study were associated with greater diameters of the largest ovarian follicle on Days 6 or 7

(Experiments 1, 2 and combined analysis), greater diameters at the time of AI (Experiment 1, 2013) and greater diameters during emergence of follicles that were classified as dominant on Day 8 (Experiment 1). These findings are consistent with the results of most (Carvalho *et al.* 2008; Dias *et al.* 2009; Peres *et al.* 2009; Martins *et al.* 2014) but not all studies (Meneghetti *et al.* 2009) that have shown that when circulating concentrations of progesterone are lower larger ovarian follicles develop during synchronisation treatments and are presented at the time of a fixed time AI. Differences in emerging follicle diameters when synchronising oestrus with progestogens and oestradiol has been suggested to be caused by a progesterone-mediated suppression of LH secretion. This is thought to reduce growth rates and dominant follicle diameters at the time of a fixed-time AI in females that have greater circulating concentrations of progesterone (Peres *et al.* 2009; Martins *et al.* 2014). Growth rates of emerging follicles that were monitored by ultrasonography in Experiments 1 and 2, did not, however, differ significantly between treatments that altered circulating concentrations of progesterone. Instead the day of emergence of follicles was delayed (Experiment 1) when concentrations of progesterone were greater or likely to be delayed in heifers treated with 1.56 g compared with 0.78 g of progesterone (Experiment 3). A delay in wave emergence in the presence of similar follicular growth rates could explain the differences in follicle diameter in association with differences in circulating concentrations of progesterone in these studies.

Edwards et al., (2013) observed similar growth rates in emerging follicles in heifers without a CL that were treated with an IVD that contained either 0.78 g or 1.56 g of progesterone. These results are, however, in contrast to those of Carvalho *et al.* (2008) who reported that lower circulating concentrations of progesterone were associated with a similar day of emergence but greater growth rates of emerging follicles. Differences between studies could be related to differences in circulating concentrations of progesterone, breeds or due to differences in interpretation of when the day of emergence occurs. Differences in the ability to resolve subtle differences in follicle growth with different ultrasound equipment or differences in the timing of when ultrasound examinations are conducted between studies could contribute to differences in interpretation of when the day of emergence occurred between studies. The reason we did not detect differences in the mean diameters of emerging follicles in heifers in Experiment 3 or growth rates between some heifers treated with different doses of progesterone could be related to the relatively small numbers of animals being monitored and the presence of heifers that failed to ovulate increasing variances around group means or that the relationship between the dose of progesterone and emerging follicle diameters is not necessarily linear.

Emergence of follicles beyond 2 to 4 mm in diameter is dependent on the secretion of gonadotrophins with wave emergence being initiated by an increase in concentrations of FSH (Adams *et al.* 1992; Bo *et al.* 1995). Any delay in wave emergence would, therefore be likely be due to suppression or delay in secretion of FSH. Delayed emergence following administration of progesterone and oestradiol has been observed in some studies in individual animals, in anoestrous animals and when different doses of progesterone were administered. Rhodes et al., (2002) observed delayed emergence in anoestrous dairy cows treated with progesterone (Rhodes *et al.* 2002) while McDougall *et al.* (2004) observed a delay in wave emergence of 2 days in anoestrous dairy cows that were treated with 6.24 g compared to 1.56 g and 3.12 g of progesterone. Suppression of FSH secretion and delayed wave emergence has been observed in some *Bos indicus* (Edwards *et al.* 2013) and *Bos*

taurus (Burke *et al.* 1996; Cavalieri *et al.* 2003) cattle treated with IVD's and oestradiol benzoate. Progesterone has not previously been shown to suppress the secretion of FSH in cattle (Price and Webb, 1988; Adams et al., 1992). This suggest that any delay in emergence observed in this study may have been related to differences in the sensitivity to oestradiol and the duration of suppression of FSH between animals associated with different circulating concentrations of progesterone or other factors. Whether or not any difference in sensitivity to oestradiol is related to the dose of progesterone administered will require further investigation.

We are unsure why differences in follicular growth rates were not observed with greater concentrations of progesterone in these studies. Decreasing circulating concentrations of progesterone have been associated in cattle with an increase in LH pulse frequency (Bergfeld *et al.* 1995; Bergfeld *et al.* 1996), greater follicular growth rates of emerging follicles (Carvalho *et al.* 2008) and larger maximal diameters of dominant follicles (Kinder *et al.* 1996; Peres *et al.* 2009). Suppression of LH pulse secretion in association with exogenous treatment with progesterone is also dose dependent (Bergfeld *et al.* 1996). Fike et al., (2004) treated postpubertal female cattle with four, incremental doses of progesterone (0.5, 1, 1.5 or 2 IVD's) and monitored changes in circulating concentrations of progesterone and the pattern of LH secretion. They found that with a greater magnitude of acute change in concentration of progesterone it took longer for re-initiation of release of LH pulses of the same amplitude as LH pulses before the change in the concentration of progesterone occurred. In the present study it was, therefore, expected that greater dose rates of progesterone would inhibit dominant follicle growth. Perhaps at the doses administered in this study LH pulse frequency was still adequate to enable uniform growth rates between treatments or a greater number of animals is required to detect differences in growth rates of follicles that emerge after administration of different doses of progesterone.

5.2 Fertility

In this study pregnancy rates to AI were variable and not always affected by follicle size or the concentration of progesterone at the time of removal of inserts. In females classified as anovulatory in Experiment 2, larger follicles and greater concentrations of progesterone at the time of removal of inserts positively influenced the odds of pregnancy to AI. In the cycling animals in Experiment 1, lower concentrations of progesterone increased the odds of pregnancy to AI in 2014 but not in 2015 while whether follicles were greater or smaller than 9 mm in diameter at insert removal did not significantly affect pregnancy rates. These differences in pregnancy outcomes between anovulatory and ovulatory females may suggest that responses may vary with ovulatory status at the time when treatments start. It also suggests that in ovulatory animals, responses can vary between years with a beneficial effect of lesser circulating concentrations of progesterone being evident in some years. Clear reasons for differences between years in the ovulatory animals are not apparent from the results of this study.

Variation in response to treatments that induce differences in circulating concentrations of progesterone have been observed across a number of other studies. Dias et al., (2009) recorded greater pregnancy rates to a timed AI when mean concentrations of progesterone in serum were 2.0 ng/mL (53.2% 42/79) compared to heifers which had mean concentrations of progesterone of 2.3 ng/mL (37.8%, 28/74) or 3.0 ng/mL (37.2%, 29/78; P <0.10). A

negative association between concentrations of progesterone during treatment with an IVD and pregnancy rates was also reported in cycling *Bos indicus* cows (Mengehetti et al., (2009) and postpubertal *Bos indicus* heifers (Peres *et al.* 2009) subjected to a timed AI. Bisinotto et al., (2015) conducted a meta-analysis of 25 studies, involving over 16,000 dairy cows and investigated the effects of supplementing dairy cows with progesterone using an IVD. Progesterone supplementation significantly increased pregnancy rates to AI by between 3 and 4% between 32 and 60 days after AI. Significant increases in pregnancy rates were, however, confined to cows that did not have a CL at the start of a synchronisation treatment or were subjected to a timed insemination without the detection of oestrus compared to cows that had a CL at the start of treatment or were detected in oestrus and inseminated before a timed AI was scheduled. Bisinotto *et al.* (2015b) found that supplementation with progesterone as part of an Ovsynch protocol reduced pregnancy rates to AI in cows that maintained their CL until administration of prostaglandin F2α (40.3% vs. 46.7%) but increased pregnancy rates to AI in those that did not have a CL at the time of administration of prostaglandin F2α (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 concentrations of progesterone before ovulation (Carvalho *et al.* 2008; Phillips *et al.* 2010; Sá Filho *et al.* 2014). When anestrous dairy cows were administered a single IVD, containing 1.56 g of progesterone or a modified IVD containing 4.7 g of progesterone, increased plasma concentrations of progesterone during treatment did not significantly affect pregnancy rates (McDougall *et al.* 2005). Potential reasons why differences in pregnancy rates have not been observed in some studies have included a failure to induce differences in concentrations of progesterone (Carvalho *et al.* 2008), or adequate secretion of LH to support follicular development during treatment in females with greater concentrations of progesterone (Sá Filho *et al.* 2014). Administration of prostaglandin F2α before the time of device removal in some studies may have also stimulated follicles to reach an adequate size before ovulation was induced (Meneghetti *et al.* 2009). In studies that have used GnRH-based protocols greater concentrations of progesterone before a timed AI improved pregnancy rates to AI by reducing the number of animals entering oestrus and ovulating and improved the synchronisation of oestrus and reduced pregnancy loss rates after AI (Bisinotto *et al.* 2015a). In this study the insemination strategy that included delaying AI to at least 72 hours after removal of inserts in females that were not detected in oestrus at 48 hours may also have allowed smaller follicles at the time of insert removal more time to increase in maturity. Administration of eCG at the time of removal of inserts has also been shown to increase follicle diameter, ovulation rates and fertility in *Bos indicus* cattle at a timed AI (Baruselli *et al.* 2004; Meneghetti *et al.* 2009; Peres *et al.* 2009). Administration of eCG may also have reduced some of the potential negative effects that high concentrations of progesterone have had on fertility in some studies by stimulating follicular development between the time of device removal and AI.

A number of studies have demonstrated consistent relationships between larger follicle diameter at insert removal and AI and pregnancy rates in *Bos indicus* (Sa Filho *et al.* 2010) and *Bos taurus* cattle (Lamb *et al.* 2001; Perry *et al.* 2005; Perry *et al.* 2007). In this study it was only in the anovulatory females where a follicle diameter of 9 mm or more on Day 6 or 7 of treatment was significantly associated with pregnancy rates to AI. Optimum diameter of potential ovulatory follicles at the time of inducing ovulation has a positive effect on the percentage of females detected in oestrus before AI, ovulation rates (Sá Filho *et al.* 2010), concentrations of progesterone after ovulation (Vasconcelos *et al.* 2001; Cavalieri *et al.*

2003), pregnancy rates to a timed AI (Mussard *et al.* 2007; Perry *et al.* 2007; Sá Filho *et al.* 2010) and reduces early embryonic loss (Perry *et al.* 2005). These benefits are associated with optimising follicle maturity and quality at the time of inducing ovulation and optimising concentrations of progesterone following ovulation. In Experiment 3 heifers that failed to ovulate had smaller diameter follicles at the time of IVD removal. Some females with smaller follicles at IVD removal in Experiment 2 may, therefore, have failed to ovulate close to the time of AI while those that did ovulate may have had follicles that were smaller than optimum diameter at the time of AI. The fact that follicle diameter on Day 6 did not significantly affect pregnancy rates to AI in the ovulatory females suggests preovulatory follicles were able to reach adequate stages of maturity before the timed AI on Day 10. The insemination strategy which delayed AI until Day 10 in those not detected in oestrus by Day 9 may also have allowed growing follicles the time required to reach an adequate preovulatory diameter.

Benefits associated with exposure to progesterone before ovulation have included increases in ovulation rates (Cavalieri *et al.* 2002; Sa Filho *et al.* 2010), improvement in oocyte quality (Fair and Lonergan 2012), increase binding to LH receptors (Khalid *et al.* 1997), alteration in the expression of angiogenic factors in large preovulatory follicles which improves subsequent luteal development and function (Christensen *et al.* 2014) and prevention of premature release of progstaglandin F2α following ovulation (Cerri et al. 2011). Improvement in fertility in anoestrous animals with higher concentrations of progesterone suggest that there is an optimal circulating concentration in these animals that facilitates beneficial effects of progesterone and/or promotes responsiveness and growth of preovulatory follicles and activity of the hypothalamic-pituitary ovarian axis. The inconsistent responses in ovulatory animals suggest that emerging follicles are responsive and grow at an adequate rate in different concentrations of progesterone but in some years fertility may be impeded when concentrations of progesterone are higher, perhaps due to a smaller dominant follicle size when ovulation is induced. It would therefore appear that in cyclic animals decreasing concentrations of progesterone before removal of IVD's is potentially advantageous to fertility when using an insemination strategy whereas anovulatory animals may benefit from greater circulating concentrations of progesterone. Further study will be needed to determine what the optimum concentration is.

In the combined analysis pregnancy rates were found to be less in the anovulatory compared with the ovulatory animal which is similar to a number of other studies (Santos *et al.* 2004; Chebel *et al.* 2006; Stevenson *et al.* 2006). Pregnancy rates were, however, only about 3% greater in the ovulatory animals (48.6%, 86/177 versus 45.4%, 64/141) suggesting that the treatments used in anovulatory animals were successful in achieving pregnancy rates in a class of animal in which delayed conception would be expected if natural breeding only had been used. The growth rates of emerging follicles were also found to be less in heifers that did and did not ovulate in Experiment 3. A number of physiological causes of reduced pregnancy rates in animals that are anoestrous at the start of synchronisation treatments have been suggested; imprecise synchronisation reducing fertilisation rates (Galvão *et al.* 2004), failure of treatment to induce ovulation and increases in embryonic loss rates (Santos *et al.* 2004). Anovulation following treatment with progesterone and oestradiol to synchronise oestrus and ovulation has been associated with smaller follicles at the time of removing inserts and at the time of the expected preovulatory LH surge (Cavalieri et al., 2002), smaller follicles with progesterone, slower growth rates, lower concentrations of oestradiol and higher concentrations of FSH (Cavalieri et al., 2003). This suggests that

follicular growth is impaired in potential anovulatory animals. The transition to puberty is associated with a progressive increase in GnRH and gonadotrophin secretion and reduced hypothalamic sensitivity to oestrogen negative feedback (Kinder *et al.* 1994; Rawlings *et al.* 2003; Atkins *et al.* 2013). A similar physiological state also is present in anoestrous cows resulting in lower frequency of release of LH secretion (Schillo 1992; Wiltbank *et al.* 2002). Reductions in IGF-1 concentrations in anoestrous cows are also thought to reduce follicular responsiveness to gonadotrophins (Spicer and Echternkamp 1995). Differences in gonadotrophin secretion, greater hypothalamic inhibition in response to secretion of oestradiol from emerging follicles and reduced responsiveness of antral follicles to gonadotrophins would explain why emerging follicles did not progress to ovulation and had lesser growth rates in some heifers (Experiment 3). Our failure to demonstrate differences in growth rates between different doses of progesterone administered to heifers in Experiment 3 may suggest that at the concentrations of circulating progesterone achieved in this study it is only the animals that have an increased risk of being anovulatory where growth rates are suppressed. There appears to be two factors that influence the diameter of potential preovulatory follicles at the time of removing inserts; circulating concentrations of progesterone which in turn affect the day of emergence of potential preovulatory follicles and the responsiveness of emerging follicles to gonadotrophins and/or the secretory pattern of gonadotrophins.

5.3 Ovulation before AI

A greater percentage of heifers in Experiment 2 in 2013 that were treated with lower concentrations of progesterone ovulated before AI (41.7%, 10/24 versus 14.8%, 4/27; P = 0.032). 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 differences were not significant. Fewer animals that were classified as being anovulatory at the start of treatment were also detected in oestrus at 48 hours after removal of inserts compared with animals classified as ovulatory. These results suggest that differences in circulating concentrations of progesterone during treatments with an IVD and differences in cyclic status can affect intervals to oestrus and ovulation which could affect the optimal timing for any fixed-time AI strategy. Optimal pregnancy rates are usually obtained when females are inseminated 5 to 17 hours after the onset of oestrus (Nebel *et al.* 2000), when concentrations of oestrogen are greater at the time of AI (Perry *et al.* 2005; Lopes *et al.* 2007) or when animals are detected in oestrus before or at the time of a fixed-time AI (Galvão *et al.* 2004; Perry *et al.* 2007; Sa Filho *et al.* 2010; Sa Filho *et al.* 2011). Using an insemination strategy that combines a period of detection of oestrus followed by a timed AI may help to reduce any variance in pregnancy rates obtained by a single timed AI although labour requirements increase.

Onset of the preovulatory LH surge usually occurs close to the time of onset of oestrus with ovulation occurring 24 to 28 hours later (Cavalieri *et al.* 1997; Sartori and Barros 2011). Few animals are normally detected in oestrus within 24 hours of removing an IVD when synchronising oestrus in cattle (Cavalieri *et al.* 2002; Cavalieri *et al.* 2004). We therefore expected that when using the insemination strategy used in this study that few animals would ovulate before AI. 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 cows ovulated before AI. The results suggest that detection of oestrus

and AI may need to occur even earlier or at more frequent intervals after IVD removal in order for some animals to be inseminated before ovulation occurs, especially when lower circulating concentrations of progesterone exist during treatment with an IVD. Additional methods may also need to be employed to increase the sensitivity of detection of oestrus. Alternatively lengthening the duration of treatment with an IVD may help reduce variance in dominant follicle diameter and onset of oestrus and ovulation, allowing improved responses to timed AI; provided that the duration of dominance of preovulatory follicles does not increase to the point where fertility is compromised (Austin *et al.* 1999; Bleach *et al.* 2004).

5.4 Antral follicle counts

Antral follicle counts (AFC) were not significantly associated with pregnancy rates to AI or at the end of the breeding period 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.* (2012) reported that cows with AFC <15 had lower pregnancy rates to first service compared with cows with AFC's of between 16 and 24 and lower odds of pregnancy at the end of the breeding season compared with cows with AFC ≥25. Final pregnancy rates in females with AFC >4 were 8% greater than those with AFC <4 but differences were not significant. These results suggest that pregnancy rates to AI using the treatment and insemination strategies applied in this study may be compromised with females with very low AFC but some recovery in fertility may occur by the end of the breeding season in animals with low AFC. Feeding diets that increase circulating concentrations of insulin can increase follicle numbers in cows (Garnsworthy *et al.* 2008; Garnsworthy *et al.* 2009) so AFC's may have improved by the end of the breeding season when dietary limitations on ovarian function are reduced. In addition, use of the synchronisation and insemination strategy that was used in this study may have reduced any influence of AFC on fertility compared to other studies in where more cows may have been bred at a spontaneous oestrus. Further study with more animals will be needed to determine if animals with very low AFC's show lower fertility over time or if any effect of low AFC on fertility in *Bos indicus* cattle may reflect nutritional or genetic differences.

Mean (±SD) AFC appeared less in this study compared to mean AFC's found in dairy cows by Mossa *et al.* (2012) (7.6 ± 3.2 versus 18.5 ± 9.0, respectively) and in studies conducted in *Bos indicus* cattle (Segerson et al., 1984; Alvarez et al., 2000; Carvalho et al., 2008; Sartori and Barros, 2011). Mean AFC's recorded in this study were, however, closer to those reported in dairy cows fed a diet that induced a low concentration of insulin in plasma (Garnsworthy *et al.* 2009). Potential reasons for differences between studies could be related to differences between breeds, diets, the methods used to determine AFC's and limited statistical power in the present study. In this study we recorded video tapes of each examination and subsequently measured follicles with electronic callipers which may not have been undertaken in some other studies. Without electronic measurement more follicles < 3 mm in diameter may have been included in the assessment of AFC. This may explain why the mean AFC was less in this study compared with those previously reported.

5.5 Concentrations of progesterone after ovulation

In each experiment concentrations of progesterone following the end of treatment did not differ significantly between treatments. In Experiment 1, on day 17 (7 to 8 days after detection of oestrus) there was a tendency for concentrations of progesterone to be less in

heifers treated with saline compared with those treated with PG (3.7 ± 0.44 versus 5.1 ± 0.58; *P* = 0.052). On Day 23 concentrations of progesterone were similar between treatments. Sampling frequency in the first 8 days following removal of IVD's may have been inadequate to detect any potential differences between treatments. The tendency for lesser concentrations of progesterone in the first 7 days after ovulation in Experiment 1 in 2015 may have been due to the smaller diameters of potential preovulatory follicles detected at IVD removal in Experiment 1. Lesser concentrations of oestradiol during pro-oestrus smaller corporal lutea and lesser circulating concentrations of progesterone occur in association with ovulation of smaller compared with larger dominant follicles (Vasconcelos *et al.* 2001; Perry *et al.* 2005). This may result in the development of smaller embryos, weaker antiluteolytic signals (Mann *et al.* 2006), premature luteoylysis (Mann and Lamming 2000) and contribute to lower pregnancy rates in females with smaller ovulatory follicles. This would provide a reason for the pregnancy rates associated with follicles < 9 mm in diameter (Experiment 2).

5.6 Limitations of the study

Differences in environments, animal age, the length of the period of natural mating and the lactational status of animals and removal of some animals before a final pregnancy test could have contributed to some of the differences observed between years in this study. Persistent drought complicated the conduct of this study but including these variables within statistical models has allowed for the assessment of treatment effects and concentrations of progesterone on dependent variables of interest while accounting for variations across years. The lack of significant effects of treatment in Experiments 1 and 2 provided an opportunity for combining data and improving statistical power, although the findings of the combined analysis were still similar to findings of each study.

6 Conclusions/recommendations

6.1 General conclusions

Maintaining greater mean concentrations of progesterone during new wave emergence before a synchronised oestrus and ovulation increases the odds of pregnancy in animals that are initially anovulatory. In ovulatory animals results are variable with lower concentrations of progesterone favouring higher pregnancy rates in some years but not in others. A delay in wave emergence rather than differences in follicular growth rates was associated with smaller diameters of emerging follicles during treatment and at the time of AI in association with administration of oestradiol and higher circulating concentrations of progesterone. We therefore modify our original hypothesis to suggest that when synchronising oestrus by insertion of an IVD and concurrent treatment with ODB, and using the insemination strategy utilised in these studies that increasing concentrations of progesterone during new wave emergence in anovulatory animals can increase the odds of pregnancy to AI at a synchronised oestrus but may have no effect or a negative effect on fertility in ovulatory animals. Low concentrations of progesterone during treatment with an IVD may also increase the percentage of heifers that ovulate before AI, when females in oestrus 48 hours after removal of inserts are inseminated and those remaining are inseminated about 24 hours later.

Variability in pregnancy rates and responses to different circulating concentrations of progesterone in *Bos indicus* females suggest that different treatment strategies may be

needed in females that are ovulatory or anovulatory at the start of treatments to optimise pregnancy rates. Females with very low AFC (≤ 4) may have lower pregnancy rates to AI but further study is needed to verify this finding and to determine if lower fertility in animals with low AFC's persists over time.

6.1.1 Future recommended studies

- Continued refinement of treatment protocols is required to optimise fertility to AI. This includes investigating whether increasing the duration of treatment with IVD's can increase the diameter of preovulatory follicles at the time of removing IVD's and the synchrony of oestrus and ovulation without reducing fertility.
- Different responses to circulating concentrations of progesterone were observed in anovulatory and ovulatory females. Further studies could be conducted to investigate whether different concentrations of progesterone before AI in females that differ in their cyclic status before commencing treatments to synchronise oestrus affects fertility at a synchronised oestrus. This includes whether routine administration of prostaglandin F2α to induce luteolysis at the time of inserting IVDs or at other times before IVD removal should be recommended in *Bos indicus* cattle.
- Use of an AI strategy that combined AI on detection of oestrus at one point in time followed by a timed AI 24 hours later may reduce some of the disadvantages to fertility associated with a timed AI but increases requirements for labour. The use of different AI strategies can be investigated when assessing responses to timed AI to determine if fertility can be enhanced and improve economic returns.
- Comparison of conception patterns and reproductive performance over time when animals are resynchronised would determine if resynchronisation can be utilised as a strategy for further improving whole-herd reproductive performance compared to a stragegy that utilises a single AI at one synchronised oestrus.
- The significance of females with a very low AFC to herd fertility is of interest and nutritional intervention, such as spike feeding before AI could be used to reduce any detrimental impact of low AFC on fertility to AI.

7 Key messages

When synchronising oestrus in *Bos indicus* cattle and AI using the treatments and insemination strategies used within this study higher circulating concentrations of progesterone are likely to improve fertility in anovulatory animals. Lower concentrations of progesterone will either improve or not detrimentally affect fertility in ovulatory females.

Treatment strategies that increase the proportion of potential preovulatory follicles ≥ 9 mm in diameter will be expected to increase fertility to AI, particularly in cows that are anovulatory at the start of treatment provided that a duration of dominance is not excessive and AI occurs at a time when preovulatory folliclular diameter is optimal.

Anovulation following treatment to synchronise oestrus is associated with lesser growth rates and smaller diameters of ovarian follicles during treatment suggesting that additional treatment strategies leading up to AI may be needed to increase ovulation rates with synchronisation protocols.

Treatment of anovulatory females with treatments that synchronise oestrus and AI can result in acceptable pregnancy rates but results are variable. Treatments could be used to reduce calving to conception intervals where animals are anovulatory at the start of the breeding season.

8 Bibliography

Adams, GP, Matteri, RL, Kastelic, JP, Ko, JCH, Ginther, OJ (1992) Association between Surges of Follicle-Stimulating-Hormone and the Emergence of Follicular Waves in Heifers. *J Reprod and Fertil* **94**, 177-188.

Atkins, JA, Pohler, KG, Smith, MF (2013) Physiology and Endocrinology of Puberty in Heifers. *Vet Clin N Am Food A* **29**, 479-492.

Austin, EJ, Mihm, M, Ryan, MP, Williams, DH, Roche, JF (1999) Effect of duration of dominance of the ovulatory follicle on onset of estrus and fertility in heifers. *J Anim Sci* **77**, 2219-2226.

Baruselli, P, Reis, E, Marques, M, Nasser, L, Bó, G (2004) The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. *Anim Reprod Sci* **82**, 479-486.

Bergfeld, EG, Kojima, FN, Cupp, AS, Wehrman, ME, Peters, KE, Mariscal, V, Sanchez, T, Kinder, JE (1996) Changing dose of progesterone results in sudden changes in frequency of luteinizing hormone pulses and secretion of 17 beta-estradiol in bovine females. *Biol Reprod* **54**, 546-553.

Bergfeld, EG, Kojima, FN, Wehrman, ME, Cupp, AS, Peters, KE, Mariscal, V, Sanchez, T, Kittok, RJ, Garcia-Winder, M, Kinder, JE (1995) Frequency of luteinizing hormone pulses and circulating 17β-oestradiol concentration in cows is related to concentration of progesterone in circulation when the progesterone comes from either an endogenous or exogenous source. *Anim Reprod Sci* **37**, 257-265.

Bisinotto, RS, Lean, IJ, Thatcher, WW, Santos, JE (2015a) Meta-analysis of progesterone supplementation during timed artificial insemination programs in dairy cows. *J Dairy Sci* **98**, 2472-2487.

Bisinotto, RS, Pansani, MB, Castro, LO, Narciso, CD, Sinedino, LDP, Martinez, N, Carneiro, PE, Thatcher, WW, Santos, JEP (2015b) Effect of progesterone supplementation on fertility responses of lactating dairy cows with corpus luteum at the initiation of the Ovsynch protocol. *Theriogenology* **83**, 257-265.

Bleach, ECL, Glencross, RG, Knight, PG (2004) Association between ovarian follicle development and pregnancy rates in dairy cows undergoing spontaneous oestrous cycles. *Reproduction* **127**, 621-629.

Bo, GA, Adams, GP, Pierson, RA, Mapletoft, RJ (1995) Exogenous Control of Follicular Wave Emergence in Cattle. *Theriogenology* **43**, 31-40.

Burke, CR, Macmillan, KL, Boland, MP (1996) Oestradiol potentiates a prolonged progesterone-induced suppression of LH release in ovariectomised cows. *Anim Reprod Sci* **45**, 13-28.

Burns, BM, Fordyce, G, Holroyd, RG (2010) A review of factors that impact on the capacity of beef cattle females to conceive, maintain a pregnancy and wean a calf-Implications for reproductive efficiency in northern Australia. *Anim Reprod Sci* **122**, 1-22.

Carvalho, JBP, Carvalho, NAT, Reis, EL, Nichi, M, Souza, AH, Baruselli, PS (2008) Effect of early luteolysis in progesterone-based timed AI protocols in Bos indicus, Bos indicus × Bos taurus, and Bos taurus heifers. *Theriogenology* **69**, 167-175.

Cavalieri, J, Coleman, C, Rodrigues, H, Macmillan, KL, Fitzpatrick, LA (2002) The effect of timing of administration of oestradiol benzoate on characteristics of oestrus, timing of ovulation and fertility in Bos indicus heifers synchronised with a progesterone releasing intravaginal insert. *Aust Vet J* **80**, 217-23.

Cavalieri, J, Hepworth, G, Fitzpatrick, LA (2004) Comparison of two estrus synchronization and resynchronization treatments in lactating dairy cows. *Theriogenology* **62**, 729-747.

Cavalieri, J, Hepworth, G, Parker, KI, Wright, PJ, Macmillan, KL (2003) Effect of treatment with progesterone and oestradiol when starting treatment with an intravaginal progesterone releasing insert on ovarian follicular development and hormonal concentrations in Holstein cows. *Anim Reprod Sci* **76**, 177-193.

Cavalieri, J, Rubio, I, Kinder, J, Entwistle, K, Fitzpatrick, L (1997) Synchronization of estrus and ovulation and associated endocrine changes in Bos indicus cows. *Theriogenology* **47**, 801-814.

Chebel, RC, Santos, JE, Cerri, RL, Rutigliano, HM, Bruno, RG (2006) Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. *J Dairy Sci* **89**, 4205-4219.

Christensen, AC, Haresign, W, Khalid, M (2014) Progesterone exposure of seasonally anoestrous ewes alters the expression of angiogenic growth factors in preovulatory follicles. *Theriogenology* **81**, 358-367.

Dias, CC, Wechsler, FS, Day, ML, Vasconcelos, JL (2009) Progesterone concentrations, exogenous equine chorionic gonadotropin, and timing of prostaglandin F(2alpha) treatment affect fertility in postpuberal Nelore heifers. *Theriogenology* **72**, 378-85.

Echternkamp, SE, Thallman, RM (2011) Factors affecting pregnancy rate to estrous synchronization and fixed-time artificial insemination in beef cattle. *J Anim Sci* **89**, 3060-3068.

Edwards, SA, Phillips, NJ, Boe-Hansen, GB, Bo, GA, Burns, BM, Dawson, K, McGowan, MR (2013) Follicle stimulating hormone secretion and dominant follicle growth during treatment of Bos indicus heifers with intra-vaginal progesterone releasing devices, oestradiol benzoate, equine chorionic gonadotrophin and prostaglandin F(2alpha). *Anim Reprod Sci* **137**, 129-36.

Evans, AC, Mossa, F, Walsh, SW, Scheetz, D, Jimenez-Krassel, F, Ireland, JL, Smith, GW, Ireland, JJ (2012) Effects of maternal environment during gestation on ovarian folliculogenesis and consequences for fertility in bovine offspring. *Reprod Domest Anim* **47 Suppl 4**, 31-37.

Folman, Y, Kaim, M, Herz, Z, Rosenberg, M (1990) Comparison of methods for the synchronization of estrous cycles in dairy cows. 2. Effects of progesterone and parity on conception. *J Dairy Sci* **73**, 2817-2825.

Fonseca, FA, Britt, JH, McDaniel, BT, Wilk, JC, Rakes, AH (1983) Reproductive traits of Holsteins and Jerseys. Effects of age, milk yield, and clinical abnormalities on involution of cervix and uterus, ovulation, estrous cycles, detection of estrus, conception rate, and days open. *J Dairy Sci* **66**, 1128-1147.

Galvão, KN, Santos, JEP, Juchem, SO, Cerri, RLA, Coscioni, AC, Villaseñor, M (2004) Effect of addition of a progesterone intravaginal insert to a timed insemination protocol using estradiol cypionate on ovulation rate, pregnancy rate, and late embryonic loss in lactating dairy cows. *J Anim Sci* **82**, 3508-3517.

Garnsworthy, PC, Fouladi-Nashta, AA, Mann, GE, Sinclair, KD, Webb, R (2009) Effect of dietary-induced changes in plasma insulin concentrations during the early post partum period on pregnancy rate in dairy cows. *Reproduction* **137**, 759-768.

Garnsworthy, PC, Lock, A, Mann, GE, Sinclair, KD, Webb, R (2008) Nutrition, metabolism, and fertility in dairy cows: 1. Dietary energy source and ovarian function. *J Dairy Sci* **91**, 3814-3823.

Ireland, J, Ward, F, Jimenez-Krassel, F, Ireland, JLH, Smith, GW, Lonergan, P, Evans, ACO, Evans, ACO (2007) Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. *Hum Reprod* **22**, 1687-1695.

Ireland, JL, Scheetz, D, Jimenez-Krassel, F, Themmen, AP, Ward, F, Lonergan, P, Smith, GW, Perez, GI, Evans, AC, Ireland, JJ (2008) Antral follicle count reliably predicts

number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biol Reprod* **79**, 1219-1225.

Jimenez-Krassel, F, Folger, JK, Ireland, JLH, Smith, GW, Hou, X, Davis, JS, Lonergan, P, Evans, ACO, Ireland, JJ (2009) Evidence That High Variation in Ovarian Reserves of Healthy Young Adults Has a Negative Impact on the Corpus Luteum and Endometrium During Estrous Cycles in Cattle. *Biol Reprod* **80**, 1272-1281.

Khalid, M, Basiouni, G, Haresign, W (1997) Effect of progesterone pre-treatment on steroid secretion rates and follicular fluid insulin-like growth factor-1 concentrations in seasonally anoestrous ewes treated with gonadotrophin releasing hormone. *Anim Reprod Sci* **46**, 69-78.

Kinder, J, Bergfeld, E, Wehrman, M, Peters, K, Kojima, F (1994) Endocrine basis for puberty in heifers and ewes. *Journal of reproduction and fertility. Supplement* **49**, 393-407.

Kinder, JE, Kojima, FN, Bergfeld, EG, Wehrman, ME, Fike, KE (1996) Progestin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. *J Anim Sci* **74**, 1424-1440.

Lamb, GC, Stevenson, JS, Kesler, DJ, Garverick, HA, Brown, DR, Salfen, BE (2001) Inclusion of an intravaginal progesterone insert plus GnRH and prostaglandin F2alpha for ovulation control in postpartum suckled beef cows. *J Anim Sci* **79**, 2253-2259.

Lopes, AS, Butler, ST, Gilbert, RO, Butler, WR (2007) Relationship of pre-ovulatory follicle size, estradiol concentrations and season to pregnancy outcome in dairy cows. *Anim Reprod Sci* **99**, 34-43.

Mann, GE, Fray, MD, Lamming, GE (2006) Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. *Vet J* **171**, 500-503.

Mann, GE, Lamming, GE (2000) The role of sub-optimal preovulatory oestradiol secretion in the aetiology of premature luteolysis during the short oestrous cycle in the cow. *Anim Reprod Sci* **64**, 171-180.

Martins, T, Peres, RF, Rodrigues, AD, Pohler, KG, Pereira, MH, Day, ML, Vasconcelos, JL (2014) Effect of progesterone concentrations, follicle diameter, timing of artificial insemination, and ovulatory stimulus on pregnancy rate to synchronized artificial insemination in postpubertal Nellore heifers. *Theriogenology* **81**, 446-453.

McDougall, S, Compton, CW, Anniss, FM (2004) Effect of exogenous progesterone and oestradiol on plasma progesterone concentrations and follicle wave dynamics in anovulatory anoestrous post-partum dairy cattle. *Anim Reprod Sci* **84**, 303-314.

McDougall, S, Compton, CW, Hanlon, DW, Davidson, PJ, Sullivan, DJ, Gore, AH, Anniss, FM (2005) Reproductive performance in anestrous dairy cows following treatment with two protocols and two doses of progesterone. *Theriogenology* **63**, 1529-1548.

McGowan, M, Fordyce, G, O'Rourke, P, Barnes, T, Morton, J, Menzies, D, Jephcott, S, McCosker, K, Smith, D, Perkins, N (2014) Northern Australian beef fertility project: CashCow. Meat & Livestock Australia Limited North Sydney No. 1925045846 Available at http://www.mla.com.au/Research-and-development/Search-RD-reports/RD-report-details/Productivity-On-Farm/Northern-Australian-beef-fertility-project-CashCow/370.

Meneghetti, M, Peres, R, Lamb, G, Vasconcelos, J (2009) Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows I: Basis for development of protocols. *Theriogenology* **72**, 179-189.

Mossa, F, Walsh, S, Butler, S, Berry, D, Carter, F, Lonergan, P, Smith, G, Ireland, J, Evans, A (2012) Low numbers of ovarian follicles≥ 3mm in diameter are associated with low fertility in dairy cows. *J Dairy Sci* **95**, 2355-2361.

Mussard, ML, Burke, CR, Behlke, EJ, Gasser, CL, Day, ML (2007) Influence of premature induction of a luteinizing hormone surge with gonadotropin-releasing hormone on ovulation, luteal function, and fertility in cattle. *J Anim Sci* **85**, 937-943.

Nebel, RL, Dransfield, MG, Jobst, SM, Bame, JH (2000) Automated electronic systems for the detection of oestrus and timing of AI in cattle. *Anim Reprod Sci* **60-61**, 713-723.

Peres, RFG, Júnior, IC, Filho, OGS, Nogueira, GP, Vasconcelos, JLM (2009) Strategies to improve fertility in Bos indicus postpubertal heifers and nonlactating cows submitted to fixed-time artificial insemination. *Theriogenology* **72**, 681-689.

Perry, GA, Smith, MF, Lucy, MC, Green, JA, Parks, TE, MacNeil, MD, Roberts, AJ, Geary, TW (2005) Relationship between follicle size at insemination and pregnancy success. *P Natl Acad Sci USA* **102**, 5268-5273.

Perry, GA, Smith, MF, Roberts, AJ, MacNeil, MD, Geary, TW (2007) Relationship between size of the ovulatory follicle and pregnancy success in beef heifers. *J Anim Sci* **85**, 684-689.

Phillips, NJ, Fordyce, G, Burns, B, Williams, P, Mayer, D, Bo, GA, McGowan, MR (2010) Does the amount of progesterone in intravaginal implants used to synchronise oestrus affect the reproductive performance of Brahman heifers artificially inseminated at a fixed time. *Reprod Domest Anim* **45**, e392-7.

Phillips, NJ, McGowan, MR, Johnston, SD, Mayer, DG (2004) Relationship between thirty post-thaw spermatozoal characteristics and the field fertility of 11 high-use Australian dairy AI sires. *Anim Reprod Sci* **81**, 47-61.

Rawlings, NC, Evans, AC, Honaramooz, A, Bartlewski, PM (2003) Antral follicle growth and endocrine changes in prepubertal cattle, sheep and goats. *Anim Reprod Sci* **78**, 259-270.

Rhodes, FM, Burke, CR, Clark, BA, Day, ML, Macmillan, KL (2002) Effect of treatment with progesterone and oestradiol benzoate on ovarian follicular turnover in postpartum anoestrous cows and cows which have resumed oestrous cycles. *Anim Reprod Sci* **69**, 139-150.

Rico, C, Fabre, S, Médigue, C, Di Clemente, N, Clément, F, Bontoux, M, Touzé, J-L, Dupont, M, Briant, E, Rémy, B (2009) Anti-müllerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. *Biol Reprod* **80**, 50-59.

Sa Filho, MF, Crespilho, AM, Santos, JE, Perry, GA, Baruselli, PS (2010) Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled Bos indicus cows. *Anim Reprod Sci* **120**, 23-30.

Sá Filho, MF, Marques, MO, L.U.Gimenes, Torres-Júnior, JRS, Batista, EOS, Carvalho, JPB, Baruselli, PS (2014) Prostaglandin treatment at the onset of norgestomet and estradiol-based synchronization protocols did not alter the ovarian follicular dynamics or pregnancy per timed artificial insemination in cyclic Bos indicus heifers. *Anim Reprod Sci* **144**, 1-5.

Sa Filho, MF, Santos, JE, Ferreira, RM, Sales, JN, Baruselli, PS (2011) Importance of estrus on pregnancy per insemination in suckled Bos indicus cows submitted to estradiol/progesterone-based timed insemination protocols. *Theriogenology* **76**, 455-463.

Sá Filho, OG, Dias, CC, Lamb, GC, Vasconcelos, JLM (2010) Progesterone-based estrous synchronization protocols in non-suckled and suckled primiparous Bos indicus beef cows. *Anim Reprod Sci* **119**, 9-16.

Santos, JEP, Thatcher, WW, Chebel, RC, Cerri, RLA, Galvão, KN (2004) The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. *Anim Reprod Sci* **82**, 513-535.

Sartori, R, Barros, CM (2011) Reproductive cycles in Bos indicus cattle. *Anim Reprod Sci* **124**, 244-250.

Schillo, KK (1992) Effects of dietary energy on control of luteinizing hormone secretion in cattle and sheep. *J Anim Sci* **70**, 1271-82.

Singh, J, Dominguez, M, Jaiswal, R, Adams, GP (2004) A simple ultrasound test to predict the superstimulatory response in cattle. *Theriogenology* **62**, 227-243.

Spicer, LJ, Echternkamp, SE (1995) The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. *Domest Anim Endocrinol* **12**, 223-245.

Stevenson, JS, Pursley, JR, Garverick, HA, Fricke, PM, Kesler, DJ, Ottobre, JS, Wiltbank, MC (2006) Treatment of cycling and noncycling lactating dairy cows with progesterone during Ovsynch. *J Dairy Sci* **89**, 2567-2578.

Vasconcelos, JL, Sartori, R, Oliveira, HN, Guenther, JG, Wiltbank, MC (2001) Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. *Theriogenology* **56**, 307-314.

Wagner, JJ, Lusby, KS, Oltjen, JW, Rakestraw, J, Wettemann, RP, Walters, LE (1988) Carcass Composition in Mature Hereford Cows: Estimation and Effect on Daily Metabolizable Energy Requirement During Winter. *J Anim Sci* **66**, 603-612.

Walsh, SW, Williams, EJ, Evans, AC (2011) A review of the causes of poor fertility in high milk producing dairy cows. *Anim Reprod Sci* **123**, 127-38.

Wiltbank, MC, Gümen, A, Sartori, R (2002) Physiological classification of anovulatory conditions in cattle. *Theriogenology* **57**, 21-52.

9 Acknowledgements

The support of Bioniche Animal Health Australia in donating IVD's and pharmaceuticals used in this project is gratefully acknowledged. The tireless help of Peter Finlay and Martin Holzwart in managing cattle and Andrew Krockenberger in making research facilities available is very much appreciated. Cynthia Gaina contributed to the conduct and analysis of data from Experiment 1 in 2014 as part of her Masters of Tropical Veterinary Science degree. Expert technical assistance was provided by Christine Archer, Scott Blyth, Nigel Breen, Kelli Hinchliffe, Rob Jack, Jeff Palpratt, Jo Penny, Virginia Simpson, and Sally Watts. Statistical advice was provided by Graham Hepworth.