Reproductive Performance of Anestrous Dairy Cows Treated with Progesterone and Estradiol Benzoate

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ABSTRACT

Anestrus is a major reproduction problem in pasture-based dairy operations that results in poorer reproductive outcomes than herdmates detected in estrus before the start of the seasonal breeding program. The objective of the current study was to assess a combined progesterone and estradiol benzoate treatment program including resynchrony with no treatment. Anestrous pasture-fed dairy cattle ($n = 756$) in 9 herds were blocked by herd and age and assigned within sequentially presented pairs of cows to be treated with an intravaginal progesterone-releasing insert for 8 d plus 2 mg of estradiol benzoate injected i.m. at insert insertion and 1 mg of estradiol benzoate injected 1 d after insert removal (d -1). Those cows detected in estrus from 0 to 3 d had a used progesterone-releasing insert reinserted for 6 d commencing on d 16 with 0.5 mg of estradiol benzoate injected i.m. 1 d after insert removal (treatment). The other cow within the pair was left as an untreated control (control). Treatment increased the risk of insemination and pregnancy by 28 d into the breeding program and resulted in conception 15 d earlier compared with controls. In contrast, treatment did not increase the risk of pregnancy after 56 d into the breeding program or at the end of the breeding season. It is concluded that treatment of anestrous dairy cattle with progesterone and estradiol benzoate combined with reinsertion of the progesterone insert resulted in earlier conception, but no difference in the final pregnancy rate compared with no treatment.

(Key words: anestrous, dairy cattle, progesterone, estradiol benzoate)

Abbreviation key: CL = corpus luteum, EB = estradiol benzoate, P4 = progesterone, RR = relative risk.

INTRODUCTION

Anestrus is a major reproduction problem within the pasture-based New Zealand dairy industry, with

approximately 20% of cows not being detected in estrus by the start of the seasonal breeding program (Rhodes et al., 2003). Anestrous cows have poorer reproductive outcomes than herdmates detected in estrus before the start of the breeding season. Cows not detected in estrus by the start of the breeding season or breeding period have lower first-service conception rates than cows inseminated at the second or later postpartum estrus (Thatcher and Wilcox, 1973; Macmillan and Clayton, 1980; Darwash et al., 1997). Cows not observed in estrus by 60 d postpartum have a greater risk of being culled than cows that have displayed estrus (Opsomer et al., 2000). Fewer anestrous cows are detected in estrus during the first 3 wk of the breeding period (55 vs. 96%) and they have longer intervals to conception (37 vs. 22 d) than cows that have displayed estrus by the start of the breeding period (Macmillan, 2002). Between 10 and 30% of cows not detected in estrus by the start of the breeding period have a palpable corpus luteum (CL). These cows have reduced pregnancy rates during the first 28 d of the breeding period (59 vs. 67%) and have greater nonpregnancy rates at the end of the breeding period (10 vs. 4%) compared with cows that were detected in estrus (McDougall and Rhodes, 1999). Progesterone (P4) delivered via an intravaginal insert is commonly used to treat anestrous cows in combination with equine chorionic gonadotropin or estradiol benzoate (EB). Treatment with P4 results in a greater proportion of the first postpartum luteal phases being of normal rather than short duration (Garcia-Winder et al., 1986) and increases the proportion of cows expressing behavioral estrus in conjunction with ovulation (McDougall et al., 1992). Equine chorionic gonadotropin was originally used in the belief that some anestrous cows did not have follicles of sufficient size to ovulate. Field studies, however, in which anestrous cows were treated with P4 and equine chorionic gonadotropin did not improve reproductive performance compared with no treatment (Galloway et al., 1987; Jubb et al., 1989) or the same treatment instituted 2 wk after the start of the seasonal breeding program (Xu et al., 1997). In addition, it was demonstrated that large dominant follicles (>10 mm) were present from

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Table 1. Herd size, number and percentage of the herd diagnosed as anestrus, the interval from calving to start of the seasonal breeding program (days postpartum) and the milk-solids yield for anestrus cows.

Herd	Herd size No. cows	Anestrus		Days postpartum			Milk solids production ³		
		No. cows	% cows	Mean	First Q ¹	Fourth Q ²	Mean	First Q	Fourth Q
1	239	44	18.4	52	40	63	1.6	1.4	1.9
2	493	115	23.3	55	43	66	1.9	1.6	2.2
3	238	25	10.5	53	41	62	1.7	1.4	2.1
4	593	166	28.0	56	45	66	1.9	1.6	2.1
5	382	113	29.6	64	53	75	1.5	1.3	1.6
6	376	35	9.3	63	54	74	1.8	1.6	2.0
7	547	133	24.3	58	45	72	1.4	1.3	1.6
8	369	44	11.9	56	47	65	1.8	1.6	2.1
9	448	96	21.4	59	46	71	1.7	1.6	2.0
Average	409.4	85.7	19.6	57.5			1.7		
Minimum	238	25	9.3	51.7			1.4		
Maximum	593	166	29.6	64.2			1.9		
SD	124.0	50.2	7.6	4.2			0.2		

¹First quartile.²Fourth quartile.³Kilograms of milk fat and kg of milk protein/cow per d.

10 d postpartum in dairy cattle even when extended periods of postpartum anestrus ensued (McDougall et al., 1995). Estradiol has been included in treatment regimens for anestrus cows because more cows were detected in estrus following treatment with P4 for 5 to 7 d followed 1 to 2 d later by EB than for P4 alone (McDougall et al., 1992). This protocol has been extensively evaluated in treating anestrus dairy cattle in New Zealand, typically resulting in 87% of cows being detected in estrus within 7 d (varying from 69 to 100% among herds) and 42% of cows conceiving to insemination during this period (varying from 27 to 62%; Rhodes et al., 2003). Use of this protocol 8 d before the planned start of the breeding period, compared with 24 d after insemination begins, results in a greater percentage of cows being detected in estrus during the first 5 d of the breeding period (89 vs. 31%), a greater pregnancy rate by d 21 of the breeding period (60 vs. 39%), and a shorter interval to conception (20 vs. 27 d; Hanlon et al., 2000).

Treatments that result in reduced concentrations of P4 for prolonged periods promote the development of persistent ovarian follicles (Sirois and Fortune, 1990; Savio et al., 1993) and reduced fertility (Mihm et al., 1994). In these cows, regression of a dominant follicle and synchronous emergence of a new wave of follicle development may be induced by treatment with GnRH analogs or EB (Roche et al., 1999; Burke et al., 2000). A field trial demonstrated that treating anestrus cows with 2 mg of EB at the start of an 8-d period of treatment with an intravaginal P4-releasing insert, compared with similar treatment of 6 d without the initial EB injection, improved pregnancy rates by 14 d after the end of treatment, but did not alter final pregnancy rates (McDougall, 2001a).

Only about 50% of treated anestrus cows not conceiving to first insemination are detected in estrus at the time that they are expected to return to estrus, 14 to 28 d after the initial treatment (McDougall, 2001a; McDougall and Loeffler, 2004). Resynchrony, following an initial 6- to 8-d treatment with P4, is accomplished by reinserting a used P4 insert for 6 to 8 d beginning at 14 to 15 d after removal of the initial P4 insert plus injecting 0, 1, or 2 mg of EB at reinsertion and 0.5 or 1 mg of EB 1 d after insert removal. Such a resynchrony treatment resulted in 70 to 80% of nonpregnant cows being detected in estrus between d 14 and 28, and an overall reduction in the nonpregnancy rate (McDougall, 2001a; McDougall and Loeffler, 2004).

Evidence that P4 and EB treatment of anestrus cows increases reproductive performance, compared with no treatment, in pasture-fed dairy cattle is lacking because no negative controls have been included in previous studies. The current study was designed to compare a current, routinely applied treatment of anestrus with no treatment.

MATERIALS AND METHODS

A total of 3685 cows from 9 seasonally calving, predominantly white clover-rye grass-fed, dairy herds in the Waikato region of New Zealand were enrolled. Herds averaged 409 cows (SD = 124; range = 238 to 593 cows; Table 1); and cows were predominantly Holstein-Friesian (78%) with some Jersey (19%) and Jersey-Friesian crossbreeds (3%). Tail paint was applied to all cows 35 d before the start of the seasonal breeding program (average start of breeding program = October 4; range = September 20 to October 11) to aid in detection of estrus (Macmillan and Curnow, 1977). Farm

Figure 1. Diagram of experimental design for treatment of anestrus dairy cows presented for veterinary examination 10 d before the start of the breeding season. P = Intravaginal progesterone-releasing insert; EB = estradiol benzoate; NIL = untreated controls.

owners identified 985 cows in which no estrus had been observed or in which tail paint had not been removed by 10 d before the start of the seasonal breeding program, or both. Reproductive tracts of the cows were palpated by a veterinarian and those without a CL were defined to be anestrus ($n = 771$; average of 19.6% of the cows within each herd; Table 1). Cows with a CL or palpable uterine or ovarian pathology were excluded. Ovarian volume was estimated on a 1-to-5 scale (Morris and Day, 1994) and BCS recorded (1-to-10 scale; Roche et al., 2004) at the time of enrollment.

Cows were blocked by herd and age (i.e., 2 yr olds vs. >2 yr olds) and then assigned within sequentially examined pairs of cows to receive an intravaginal P4-releasing insert (CIDR-B, Pfizer Animal Health, Auckland, New Zealand) for 8 d with 2 mg of EB (CIDRIOL, Bomac Laboratories, Manukau City, New Zealand) injected i.m. at insert insertion and 1 mg of EB injected 1 d after insert removal (d -1). Those cows detected in estrus between d 0 and 3 had a used P4-releasing insert reinserted for period of 6 d commencing on d 16 with 0.5 mg of EB injected i.m. 1 d after insert removal (treatment). The other cow within the pair was left as an untreated control (control; Figure 1). After initial usage, the P4-releasing inserts were placed in a large bucket, hosed with a high-pressure hose, soaked for 24 h in a 1:200 solution of commercial disinfectant and cleanser (Virkon S, Antec International, Suffolk, UK), rinsed, and then air dried before reuse. Treatment and control cows were managed as a single group within each herd and no difference in nutritional or other management occurred between groups.

Cows detected in estrus during the subsequent 3 to 7 wk (depending on herd policy) were submitted for AI. Following this AI period, bulls were introduced to the herds for a period of 6 to 12 wk.

Pregnancy status and stage of gestation in pregnant cows was estimated for all cows ($n = 3685$) approximately 11 wk after the planned start of the seasonal breeding program and again 6 wk after the bulls were finally removed from the herd. Transrectal ultrasonography was used to estimate the stage of gestation (Curran et al., 1985; White et al., 1985) at each examination and the day of conception calculated (i.e., calendar date of examination minus estimated number of days pregnant). Where this estimated day of conception was within 7 d of a recorded AI or bull breeding, the recorded breeding date was accepted as the day of conception. Where the estimated date deviated >7 d from the last recorded date, the estimated date was used as the conception date in subsequent analyses. Cows that died or were removed from the herd before pregnancy diagnosis were included in submission rate analyses up to the date of culling, but removed from pregnancy rate analyses and right-censored in survival analyses.

Volume of milk produced by each cow was estimated 3 or 4 times during lactation by inclusion of a milk meter in the long milk line. A consistent fraction of milk was collected from a sequential evening and morning milking, the samples weighed, and a subset of the combined evening and morning milking taken for infrared analysis of milk fat and protein concentration and SCC.

Data including age (yr), calving date, calving type (i.e., normal, aborted, induced, or assisted), number of calves born, breed, milk production [milk solids (i.e., milk fat and milk protein; kg/cow per d), milk fat percentage, milk protein percentage, and SCC], ovarian status at examination (i.e., CL detected or not detected), uterus status at examination (i.e., normal, enlarged, or adhesions), BCS at d -10, all recorded AI

and natural breeding dates, any disease diagnoses (including date of diagnoses and treatments), removal date and reason, and pregnancy diagnoses were retrieved either from a database (Livestock Improvement Corporation, Hamilton, New Zealand), veterinary records, or from herd records.

Statistical Analyses

Balance of treatments for age, milk yield and composition, BCS, ovarian score, interval from calving to start of the breeding program, the total length of the breeding program were compared using *t*-tests and the breed distribution between the groups by χ^2 .

Outcome variables of interest were probability of insemination by d 21 and 28 after the start of the breeding program (i.e., number of cows inseminated by d 21 or 28 ÷ total number of enrolled cows), the probability of conception at the first insemination within 24 d of the start of the breeding program (i.e., number of cows confirmed pregnant to insemination by d 24 ÷ total number of cows inseminated by d 24), the probability of conception by d 28 and 56 after the start of the breeding program (i.e., number of cows confirmed pregnant by d 28 or 56 ÷ total number of enrolled cows), and the probability of being diagnosed pregnant approximately 6 wk after the end of the breeding period.

The predictor variable of main interest was treatment (i.e., P4 + EB treatment with reinsertion). However, several other variables of biological interest were assessed to increase understanding of their effects on reproductive outcomes in anestrus cows, and to control for any confounding of these variables on the treatment effect. These variables were herd, age at calving, breed (categorized as Friesian or Jersey if that breed was >11/16th of the cow's genetics; all others were categorized as crossbred), BCS (1-to-10 scale), sum of ovary scores (i.e., left and right score on a 1-to-5 scale), days from a calving to the onset of the breeding program, and the milk yield from a test immediately preceding, or if that was unavailable, following the start of AI period; for total milk volume, milk fat and protein percentages. Continuous predictor variables were categorized into quartiles or meaningful groups for categorical analyses. Farm-recorded disease records in all herds from calving to the end of the breeding period were included as predictors, and grouped into 5 categories denoting the cumulative incidence (absence or presence) of peripartum disease (retained fetal membranes, dystocia, metritis, and endometritis), and pre- and post-onset of breeding mastitis or lameness. An individual cow could be included in more than one disease category.

Cows that were confirmed pregnant at the first pregnancy diagnosis, but were subsequently found to be either not pregnant or be pregnant to a later breeding ($n = 3$), had the first conception taken as the time to conception for all analyses, except for the probability of being pregnant at the final test following the end of the breeding period.

Fifteen cows were excluded from analyses in multivariable models because of missing herd test results, and one because of a nonsensible test result. The final data set for multivariable analysis of all outcome variables, except probability of conception at first service, consisted of 755 cows. Probability of conception at first AI within 24 d of the onset of the breeding season was estimated by analysis of 599 cows, and its multivariable analysis was carried out using 588 cows with no missing herd test records, due to cows not being inseminated during this period, missing milk production or other data.

Univariate analyses were performed on all variables to check for outlying values and inconsistencies. Outcome and predictor categorical variables were analyzed by Fisher's exact test for categorical data, and from this, relative risk (RR) was calculated for the crude associations. Mantel-Haenszel adjusted RR was calculated for the same associations to check for confounding of the associations by other variables. In subsequent multivariable models, RR was calculated for categorical variable coefficients by the method of Beaudeau and Fourichon (1998), and for continuous variables by the method of Zhang and Yu (1998). Relative risk was used as the measure of association because the rare outcome approximation of odds ratio to RR could not be used in this study, and risk was considered a more appropriate measure with the prospective data used. Statistical significance was set at a level of $P \leq 0.05$ for all analyses.

Multivariable logistic regression was used to test the effect of treatment and other variables of interest on the RR of insemination by d 21 and 28, conception at first insemination by d 24, and pregnancy by d 28 and 56, and at the end of breeding season. Model building was by manual forward step-wise addition of variables previously significant in bivariate or stratified analysis at $P < 0.2$, with variables retained in the model when likelihood ratio tests were significant at $P \leq 0.05$, or if they caused a change of >15% in the coefficient of the treatment variable. Models were checked for missing potentially important variables using automated backward stepwise regression on the full set of variables. Biologically meaningful interactions between treatment and other variables were tested. Diagnostic plots of residuals and influence mea-

Table 2. Estimates of crude (univariate) means and crude relative risks (RR) of reproductive outcomes for anestrus dairy cows treated with an intravaginal progesterone (P4)-releasing insert for 8 d with injection of 2 mg and 1 mg of estradiol benzoate at insert insertion and 1 d after removal, respectively, and by reinsertion of a used P4 insert compared with untreated controls.

Variable	Count		Mean effect	RR	95% CI	
	No	Yes			Low	High
Estrus by d 28						
Control	116	272	0.70			
Treated	28	356	0.93	1.32	1.23	1.42
Conceived at first AI						
Control	137	114	0.45			
Treated	184	165	0.47	1.04	0.87	1.24
Pregnant by d 28						
Control	251	137	0.35			
Treated	171	213	0.55	1.57	1.34	1.85
Pregnant by d 56						
Control	132	256	0.66			
Treated	115	269	0.70	1.06	0.96	1.17
Pregnant at end of breeding program						
Control	60	328	0.85			
Treated	55	329	0.86	1.02	0.96	1.08

tures were assessed for unusual patterns or influential subjects.

Survival analysis techniques were used to investigate the effect of treatment on days to conception. Initially, Kaplan-Meier survival curves were calculated for the categorical variables, and variables with significant log-rank tests at levels of $P < 0.2$ kept for multivariable modeling. Daily hazard of conception was estimated by a multivariable Cox's proportional hazards model. Model building was in a forward manual step-wise manner, with variables retained in the model when likelihood ratio statistic was $P \leq 0.05$, or when they caused a change of $>15\%$ in the coefficient of the treatment variable. Proportionality of hazards assumption was checked by assessing a plot of scaled Schoenfeld residuals for each variable (Therneau, 1994). Because the treatment variable did not meet this assumption, a time-dependent covariate model was fitted. The final model deviance residuals and influence measures also were checked for unusual patterns or influential subjects. Smoothing splines were fitted to the final simple Cox's model for the terms calving to onset of AI and milk protein percentage at first herd test and these were plotted to display the effect of these variables on the daily hazard of conception.

Data was stored in a Microsoft Access database and statistical analysis was carried out in "R 1.9.0—A language and an environment" (The R Project for Statistical Computing, <http://www.r-project.org>).

Power Statistics

It was calculated a priori that to detect a 10% difference in the proportion of cows pregnant by d 56, ap-

proximately 300 cows per treatment were necessary ($\alpha = 0.05$, $\beta = 0.2$; assuming the control achieved 70% pregnancy rate by d 56 of the breeding season; PASS, Number Cruncher Statistical Systems, Kaysville, UT; www.ncss.com). To account for cows lost to follow up, the aim was to enroll approximately 400 cows per treatment.

The study was undertaken following the approval of the Animal Ethics Committee of AgResearch, Ruakura, Hamilton, New Zealand.

RESULTS

No differences were detected between control and treatment cows for age (4.5 vs. 4.6 yr), BCS (3.6 vs. 3.6), ovary score (3.5 vs. 3.5), days from calving to start of the seasonal breeding program (58.2 vs. 57.6), total duration of breeding program (116 vs. 116 d), milk yield (22.1 vs. 22.0 L/cow per d), milk fat percentage (4.4 vs. 4.3%), milk protein percentage (3.4 vs. 3.4%) or breed, respectively.

Cumulative incidence of herdowner-reported peripartum diseases, prestart of breeding clinical mastitis, poststart of breeding clinical mastitis, prestart of breeding lameness, and poststart of breeding lameness were 3.6% ($n = 27$), 10.3% ($n = 78$), 2.4% ($n = 18$), 1.7% ($n = 13$), and 4.9% ($n = 37$), respectively.

Univariate Analyses

Treatment increased the risk of cows being inseminated by d 28 and increased the risk of pregnancy by d 28 (Table 2). The risk of conception following first insemination and the risk of pregnancy by d 56 or by

Figure 2. Survival analysis (Kaplan-Meier) for conception following the onset of the breeding season for anestrus dairy cows treated with either progesterone and estradiol benzoate (dashed line) or untreated control cows (solid line).

the end of the breeding season did not differ between treatments (Table 2).

Median interval from the start of the breeding program to conception was shorter ($P = 0.06$) for treated than control cows [40 d (95% confidence interval = 36 to 47 d) vs. 25 d (95% confidence interval = 24 to 43 d)], respectively; Figure 2).

Multivariate Analyses

Submission rates by d 28. Treatment increased the risk of insemination by d 28 (RR = 1.39; Table 3). Probability of insemination also was affected by age, BCS, ovarian volume, days from calving to the start of the breeding season, milk yield, milk protein percentage, and herd (Table 3).

Risk of conception to insemination by d 24. Treatment did not affect the risk of conception to insemination by d 24 (RR = 1.06; Table 4). Conception rate was affected by age, ovarian volume, days from calving to the onset of the breeding season, and herd (Table 4).

Pregnancy rate by d 28. Treatment increased the risk of pregnancy by d 28 (RR = 1.66; Table 5). Probability of pregnancy by d 28 was also affected by ovarian volume, days from calving to the onset of the breeding season, milk protein percentage, and herd (Table 5).

Pregnancy rate by d 56. Treatment did not increase the risk of pregnancy by d 56 (RR = 1.07; Table 6). Probability of pregnancy by d 56 was affected by the

Figure 3. Daily hazard of conception from the final Cox's proportional hazards model for A) days from calving to the onset of the breeding season and B) milk protein percentages.

interval between calving and the start of the breeding season, milk protein percentage, and herd (Table 6).

Final pregnancy rate. Treatment did not increase the risk of final pregnancy within the breeding season (R = 1.02; Table 7). Probability of pregnancy by the end of the breeding season was affected by days from calving to the onset of the breeding season, milk protein percentage, and herd (Table 7).

Table 3. Final general linear model of the probability and relative risks (RR) of cows being inseminated by d 28 for anestrus dairy cows treated with an intravaginal progesterone (P4)-releasing insert for 8 d with injection of 2 mg and 1 mg of estradiol benzoate at insert insertion and 1 d after removal, respectively and by reinsertion of a used P4-releasing insert compared with untreated controls.

Predictor variable	Limits	Coeff. estimate	SE	P	RR	95% CI ¹		Mean effect	
						Low	High	Control	Treated
Treatment	Control (Ref) ²							0.70	
	Treated	2.09	0.26	0.00	1.39	1.31	1.45		0.93
Age, yr	2 (Ref)							0.64	0.88
	3 to 4	0.75	0.33	0.02	1.13	1.02	1.23	0.67	0.93
	4 to 5	1.34	0.44	0.00	1.19	1.08	1.24	0.81	0.97
	>6	0.44	0.40	0.28	1.08	0.93	1.18	0.73	0.95
BCS ³	<3.5 (Ref)							0.61	0.90
	3.5	0.44	0.34	0.20	1.08	0.96	1.19	0.73	0.90
	4 to 4.5	0.86	0.37	0.02	1.15	1.03	1.26	0.74	0.97
Ovary score ⁴	2 (Ref)							0.51	0.87
	3	0.78	0.29	0.01	1.14	1.04	1.23	0.71	0.91
	4	1.23	0.36	0.00	1.19	1.09	1.25	0.76	0.97
	5 to 9	1.23	0.42	0.00	1.18	1.07	1.23	0.77	0.97
Days postpartum	31 to 45 (Ref)							0.63	0.90
	46 to 58	0.76	0.30	0.01	1.13	1.03	1.20	0.69	0.92
	59 to 69	1.21	0.33	0.00	1.18	1.10	1.25	0.75	0.97
	>70	0.97	0.33	0.00	1.16	1.06	1.22	0.74	0.92
Milk yield, L/cow per d		-0.06	0.03	0.06	0.98	0.97	1.00	0.69	0.96
Milk protein, %	2.3 to 3.2 (Ref)							0.60	0.89
	3.2 to 3.4	0.04	0.29	0.88	1.01	0.90	1.10	0.61	0.92
	3.4 to 3.6	0.90	0.31	0.00	1.15	1.05	1.22	0.81	0.93
	>3.6	1.07	0.35	0.00	1.17	1.07	1.24	0.75	0.99

¹Confidence interval.

²Ref = Reference category.

³BCS on a 1-to-10 scale (Roche et al., 2004).

⁴Sum of the left and right ovary score, an estimate of ovarian volume on a 1-to-5 scale (Morris and Day, 1994).

Table 4. Final general linear model of the probability and relative risk (RR) of conception to first insemination by d 24 for anestrus dairy cows treated with an intravaginal progesterone-releasing insert for 8 d with injection of 2 mg and 1 mg of estradiol benzoate at insert insertion and 1 d after removal, respectively and by reinsertion of a used progesterone-releasing insert compared with untreated controls.

Predictor variable	Limits	Coeff. estimate	SE	P	RR ¹	95% CI ¹		Mean effect	
						Low	High	Control	Treated
Treatment	Control (Ref)							0.45	
	Treated	0.10	0.18	0.57	1.06	0.88	1.28		0.47
Age, yr	2 (Ref)							0.59	0.49
	3 to 4	-0.27	0.24	0.26	0.86	0.66	1.11	0.41	0.46
	5 to 6	0.00	0.28	1.00	1.00	0.73	1.32	0.48	0.57
	>6	-0.85	0.30	0.00	0.60	0.40	0.86	0.33	0.39
Ovary score ³	2 (Ref)							0.39	0.37
	3	0.50	0.27	0.06	1.30	0.98	1.68	0.50	0.48
	4	0.76	0.30	0.01	1.46	1.10	1.86	0.46	0.57
	5 to 9	0.34	0.33	0.30	1.19	0.84	1.58	0.38	0.43
Days postpartum	31 to 45 (Ref)							0.42	0.38
	46 to 58	-0.03	0.25	0.90	0.98	0.74	1.26	0.37	0.47
	59 to 69	0.50	0.25	0.04	1.29	1.01	1.61	0.58	0.54
	>70	0.03	0.26	0.91	1.02	0.76	1.31	0.42	0.51

¹Confidence interval.

²Ref = Reference category.

³Sum of the left and right ovary score, an estimate of ovarian volume on a 1 to 5 scale (Morris and Day, 1994).

Table 5. Final general linear model of the probability and relative risk (RR) of pregnancy by d 28 of the seasonal breeding program for anestrous dairy cows treated with an intravaginal progesterone-releasing insert for 8 d with injection of 2 mg and 1 mg of estradiol benzoate at insert insertion and 1 d after removal, respectively and by reinsertion of a used progesterone releasing insert compared with untreated controls.

Predictor variable	Limits	Coeff. estimate	SE	P	RR	95% CI ¹		Mean effect	
						Low	High	Control	Treated
Treatment	Control (Ref) ²								
	Treated	0.92	0.16	0.00	1.66	1.40	1.97	0.36	0.55
Ovary score ³	2 (Ref)							0.21	0.44
	3	0.70	0.23	0.00	1.45	1.15	1.80	0.43	0.57
	4	0.86	0.25	0.00	1.53	1.22	1.87	0.39	0.66
	5 to 9	0.36	0.27	0.19	1.20	0.90	1.52	0.29	0.50
Days postpartum	31 to 45 (Ref)							0.31	0.45
	46 to 58	0.22	0.22	0.33	1.12	0.88	1.40	0.28	0.59
	59 to 69	0.63	0.23	0.01	1.38	1.10	1.67	0.46	0.59
	>70	0.53	0.23	0.02	1.31	1.04	1.61	0.38	0.60
Milk protein, %	2.3 to 3.2 (Ref)							0.28	0.45
	3.2 to 3.4	0.36	0.22	0.10	1.21	0.96	1.49	0.31	0.59
	3.4 to 3.6	0.68	0.23	0.00	1.41	1.13	1.71	0.46	0.59
	>3.6	0.52	0.24	0.03	1.30	1.02	1.62	0.36	0.59

¹Confidence interval.

²Ref = Reference category.

³Sum of the left and right ovary score, an estimate of ovarian volume on a 1 to 5 scale (Morris and Day, 1994).

Cox's proportional hazards models. Treatment increased ($P < 0.01$) the hazard of pregnancy over the first 24 d of the seasonal breeding program (HR = 2.18; Table 8), but decreased ($P = 0.04$) the hazard of pregnancy after d 24 (HR = 0.80; Table 8). Hazard of pregnancy was affected by age, ovary score, the interval between calving and the start of the seasonal breeding program (Figure 3A), milk protein percentage (Figure 3B), and herd (Table 8).

DISCUSSION

Treatment of anestrous cows resulted in more cows being inseminated and pregnant by 28 d into the breed-

ing program and a shorter interval from the start of the breeding program to conception than untreated control cows in the present study.

This is the first study to directly demonstrate a comparative benefit in treating pasture-fed, anestrous dairy cattle. Previous negative-control designed studies have either failed to demonstrate any difference between treated and control cows (Jubb et al., 1989) or demonstrated only short-term (i.e., during the first 14 d after treatment) differences (Galloway et al., 1987). In a previous study, treatment did not increase the proportion of cows in estrus or those conceiving during 21 and 42 d after insert insertion (Jubb et al.,

Table 6. Final general linear model of the probability and relative risk (RR) of pregnancy by d 56 of the seasonal breeding program for anestrous dairy cows treated with an intravaginal progesterone-releasing insert for 8 d with injection of 2 mg and 1 mg of estradiol benzoate at insert insertion and 1 d after removal, respectively, and by reinsertion of a used progesterone-releasing insert compared with untreated controls.

Predictor variable	Limits	Coeff. estimate	SE	P	RR	95% CI ¹		Mean effect	
						Low	High	Control	Treated
Treatment	Control (Ref) ²								
	Treated	0.23	0.16	0.17	1.07	0.97	1.19	0.66	0.70
Days postpartum	31 to 45 (Ref)							0.58	0.57
	46 to 58	0.43	0.22	0.05	1.14	1.00	1.26	0.61	0.71
	59 to 69	0.84	0.24	0.00	1.26	1.12	1.38	0.73	0.75
	>70	1.00	0.25	0.00	1.30	1.16	1.42	0.74	0.79
Milk protein, %	2.3 to 3.2 (Ref)							0.56	0.60
	3.2 to 3.4	0.44	0.22	0.05	1.14	1.00	1.27	0.65	0.70
	3.4 to 3.6	0.94	0.24	0.00	1.28	1.15	1.40	0.73	0.79
	>3.6	0.85	0.25	0.00	1.26	1.11	1.38	0.72	0.73

¹Confidence interval.

²Ref = Reference category.

Table 7. Final general linear model of the probability and relative risk (RR) of pregnancy by the end of the seasonal breeding program for anestrus dairy cows treated with an intravaginal progesterone releasing insert for 8 d with injection of 2 mg and 1 mg of estradiol benzoate at insert insertion and 1 d after removal, respectively, and by reinsertion of a used progesterone-releasing insert compared with untreated controls.

Predictor variable	Limits	Coeff. estimate	SE	P	RR ¹	95% CI ¹		Mean effect	
						Low	High	Control	Treated
Treatment	Control (Ref) ²							0.85	
	Treated	0.14	0.22	0.51	1.02	0.96	1.08		0.86
Days postpartum	31 to 45 (Ref)							0.79	0.76
	46 to 58	0.85	0.29	0.00	1.11	1.04	1.16	0.83	0.89
	59 to 69	1.13	0.31	0.00	1.13	1.07	1.17	0.86	0.91
	>70	1.31	0.34	0.00	1.15	1.08	1.18	0.94	0.89
Milk protein, %	2.3 to 3.2 (Ref)							0.80	0.80
	3.2 to 3.4	0.27	0.28	0.32	1.04	0.96	1.10	0.81	0.84
	3.4 to 3.6	1.24	0.34	0.00	1.14	1.08	1.18	0.88	0.96
	>3.6	1.11	0.33	0.00	1.13	1.06	1.17	0.91	0.84

¹Confidence interval.

²Ref = Reference category.

1989). In that study, only 65 and 77% of treated cows and 50 and 82% of control cows were detected in estrus by 14 and 35 d after insert removal, respectively (Jubb et al., 1989). In comparison, 93 and 70% of treated and control cows were detected in estrus by 29 d after insert removal, respectively, in the present study. Others studies have compared P4 and EB treatments commencing either 8 to 10 d before ("early" treatment), or

16 d after ("late" treatment) the start of the seasonal breeding program (Xu et al., 1997; Hanlon et al., 2000). Early treatment resulted in more cows pregnant by 21 d (Hanlon et al. 2000), and a shorter interval (Hanlon et al., 2000), or no difference in the interval (Xu et al., 1997 to conception compared with late treatment of cows. Variation in the study populations or in the treatment protocols may account for differences in out-

Table 8. Final Cox's proportional hazards model for the interval from the start of the seasonal breeding program to conception for anestrus dairy cows treated with an intravaginal progesterone releasing insert for 8 d with injection of 2 mg and 1 mg of estradiol benzoate at insert insertion and 1 d after removal, respectively, and by reinsertion of a used progesterone releasing insert compared with untreated controls.

Predictor variable	Limits	Coeff. estimate	Coeff. SE	P	Hazard ratio	95% CI ¹	
						Low	High
Treatment	≤24 d	0.78	0.12	0.00	2.18	1.73	2.75
	>24 d	-0.23	0.11	0.04	0.80	0.64	0.99
Age, yr	2 (Ref) ²						
	3 to 4	0.05	0.11	0.63	1.05	0.85	1.30
	5 to 6	-0.07	0.13	0.61	0.93	0.72	1.22
	>6	-0.45	0.14	0.00	0.64	0.49	0.84
Ovary score ³	2 (Ref)						
	3	0.40	0.12	0.00	1.49	1.18	1.87
	4	0.54	0.13	0.00	1.72	1.33	2.24
	5	0.40	0.15	0.01	1.49	1.11	2.01
Days postpartum ⁴	31 to 45 (Ref)						
	46 to 58	0.27	0.12	0.02	1.31	1.04	1.64
	59 to 69	0.51	0.12	0.00	1.66	1.31	2.10
	>70	0.52	0.12	0.00	1.68	1.32	2.14
Milk protein, %	2.3 to 3.2 (Ref)						
	3.2 to 3.4	0.18	0.12	0.12	1.20	0.95	1.50
	3.4 to 3.6	0.49	0.12	0.00	1.63	1.30	2.05
	>3.6	0.49	0.12	0.00	1.63	1.28	2.07

¹Confidence interval.

²Ref = Reference category.

³Sum of the left and right ovary score, an estimate of ovarian volume on a 1 to 5 scale (Morris and Day, 1994).

⁴Days from calving to the onset of the breeding season.

comes among studies. Treatment differences included route of delivery and type of progestogen (Galloway et al., 1987), use of equine chorionic gonadotropin at the time of insert removal rather than EB 1 d later (Galloway et al., 1987; Jubb et al., 1989; Xu et al., 1997), and no reinsertion of the P4-releasing insert (Galloway et al., 1987; Xu et al., 1997) or reinsertion only in those animals not detected in estrus by 14 d after insert removal (Jubb et al., 1989; Hanlon et al., 2000). In addition, cows were enrolled only when >44 d (Galloway et al., 1987), >35 d (Jubb et al., 1989) or >30 d (Hanlon et al., 2000) postpartum compared with >25 d in the current study. Longer postpartum intervals are associated with an increased risk of being detected in estrus, insemination, and conception. Thus, in studies in which early postpartum cows are excluded, controls are likely to have reproductive performance closer to that of treated cows compared with studies in which cows are enrolled earlier postpartum. This is demonstrated by the fact that the untreated controls in a previously designed study having negative controls (Jubb et al., 1989) had relatively greater estrus-detection rates than controls in the current study. When controls have more risk of expressed estrus, conception, and pregnancy, the probability that treatment will improve reproductive performance becomes less likely.

Hormonal synchrony treatments have reduced conception rates in some studies of estrus-cycling (Xu et al., 1996; Jobst et al., 2002) and anestrus cows (Hanlon et al., 2000), but not in other studies of cycling (Xu and Burton, 2000) and anestrus cows (Jubb et al., 1989; Xu et al., 1997) compared with cows inseminated at spontaneous estrus. No difference in conception rates between treated and control cows occurred in the current study. First-service conception rate in these anestrus cows, however, was less (45%) than that reported for cycling cows inseminated following detection of spontaneous expression of estrus (64%; Xu et al., 1996) or following a synchrony protocol (64%; Xu and Burton, 2000), and undertaken under similar farm systems as the current study. Thus, reduced conception rates in anestrus cows are not due to the treatment protocol, but rather to some underlying differences in the physiology of cows inseminated at their first postpartum estrus.

Reinsertion of the used P4-releasing insert (resynchrony) was included in the current study protocol because resynchrony has been shown to improve the probability of pregnancy compared with nonresynchronized cows by d 28 of the breeding season (McDougall and Loeffler, 2004). However, resynchrony has been associated with reduced first-service conception rate in some studies of cycling (Chenault et al., 2003) and

previously anestrus cows (McDougall, 2001a; McDougall, 2003), but not in other studies of cycling (El-Zarkouny and Stevenson, 2004) and anestrus (McDougall and Loeffler, 2004) cows. The resynchrony protocol used in the current study was one that was previously found to not reduce first-insemination conception rates (McDougall and Loeffler, 2004). No difference was detected in the first-insemination conception rate between treated and control cows in the current study, suggesting that resynchrony did not reduce conception rate in the treated cows.

Treated anestrus cows conceived approximately 15 d earlier than untreated control cows. Earlier conception is likely to result in a longer subsequent lactation, because in seasonally calving herds, all cows tend to cease lactation on a single calendar day, irrespective of calving date. In addition, earlier calving relative to the start of the seasonal breeding program in the subsequent season may result in improved reproduction performance because more days between calving and the onset of the breeding season is associated with increased risk for estrus, insemination, conception, and pregnancy. A full economic analysis of this treatment protocol remains to be undertaken.

Hazard of pregnancy following treatment changed over time. Testing of the Schoenfeld residuals found that the hazard was not consistent across time, which violates the basic assumption of Cox's proportional hazards models that the ratio of hazards is consistent over time. The fitting of a time-dependent treatment covariate resulted in the proportional hazards assumptions being met and validating use of the Cox's proportional hazards model. The hazard of pregnancy up to d 24 following treatment was 2.8. In other words, on any day (up to d 24), treated cows were 2.8 times more likely to become pregnant than were controls. After d 24, however, treated cows had a hazard of pregnancy of 0.8 and this was less than that for control cows. This smaller hazard indicates that treated cows were less likely to become pregnant after d 24. A greater proportion of the treated than control cows were pregnant by d 28 (i.e., 55 vs. 35% of treated vs. control cows, respectively). About 15% of both treated and control cows were not pregnant at the end of the breeding season. If these nonpregnant cows were regarded as subfertile or infertile, then the proportion of infertile cows within the treatment at d 24 was relatively greater than in the control. That is, of the 45% of cows not pregnant in the treatment group by d 28 of the breeding program, 33% of these may be infertile (i.e., 15%/45%). Conversely, 15%/65% or 23% may be sub- or infertile in the control group at this time. Thus, earlier conception in the treated cows resulted in fewer nonpregnant cows by d 24, but a greater

proportion of potentially sub- or infertile treated cows. Direct effects of the treatment on subsequent probability of expression of estrus or of conception after d 24 cannot be ruled out. No mechanism, however, for these reduced traits is apparent, so further investigation is warranted.

A number of other variables were associated with reproductive performance in these anestrus cows. Greater milk protein percentages are associated with increased probability of estrus and pregnancy by d 28, d 56, and at the end of the breeding season after treatment of anestrus cows (McDougall, 2003), and with increased d-21 insemination and d-42 pregnancy rates in cycling cows (Buckley et al., 2003). Milk protein percentages can be influenced by greater proportions of concentrate in the diet (Spiekens et al., 1991; Petit et al., 2001), but is unaffected by dietary fat supplementation (Ferguson et al., 1990). Substitution of fish meal for other protein sources increased in milk protein percentages in some age groups, but greater protein yield did not alter reproductive performance (Carroll et al., 1994; Burke et al., 1997). It is not clear whether the differences in milk protein percentage and the association between milk protein percentage and reproduction are due to genetic differences among cows in milk protein percentage or due to differences in nutrient intake and cow or herd management that lead to changes in milk protein percentages.

Ovarian score is used clinically by veterinarians to predict onset of ovulation and estrus in New Zealand. Ovary score has been correlated with in vitro ovarian volume (Morris and Day, 1994) and was positively associated with BCS in the present study (data not presented). Smaller ovary score was associated with lesser likelihood of being detected in estrus by d 28, a reduced likelihood of conceiving to insemination, and a reduced pregnancy rate by d 28 in the current study. A previous study reported a relationship between ovary score and probability of estrus, but not between ovary score and response to treatment or between ovary score and BCS (Nation et al., 1998). Smaller ovary scores may be associated with fewer large follicles being present in the ovary. The maximum diameter of the largest ovarian follicle in anestrus cows increases with sequentially forming dominant follicles in cows that have more than one dominant follicle before first postpartum ovulation (McDougall et al., 1995). Thus, larger ovary scores may be associated with cows closer to spontaneous resumption of ovulation and estrus or with presence of a CL not detected by manual palpation of the ovary.

Poor BCS is associated with reduced probability of being detected in estrus of treated anestrus cows by d 5 or 14 (Hanlon et al., 2000; McDougall and Loeffler,

2004), and a reduced likelihood pregnancy by d 28, d 56, and the end of breeding season (McDougall and Loeffler, 2004). In the current study, BCS was associated with the probability of being detected in estrus by d 21. In the models of pregnancy rate, BCS was not significant. However, the collinearity of BCS with ovarian score (data not presented) may have resulted in BCS being removed from the models.

Peripartum diseases and clinical mastitis adversely affect reproductive performance of dairy cattle (Barker et al., 1998; Fourichon et al., 2000; Gröhn and Rajala-Shultz, 2000; McDougall, 2001b). In the present study, however, none of the disease categories was included in any of the final models. This may be due to other predictors (e.g., milk volume or age), correlated with probability of peripartum diseases, being more strongly associated with reproductive outcomes than diseases themselves. Alternatively, the small incidence of diseases may have reduced the probability that a relationship could be detected in the current study.

No significant interactions were detected (i.e., treatment $\times$ herd, treatment $\times$ age, treatment $\times$ BCS, treatment $\times$ ovary score, or treatment $\times$ milk yield, or composition interactions) in any of the models. No interactions indicate that treatment effects were consistent across the range of herds, ages, milk yields, ovary scores, and BCS in the population in which the study was conducted.

CONCLUSIONS

It is concluded that treatment of anestrus, pastured dairy cows with P4 and EB with subsequent resynchronization of estrus via a used P4-releasing insert plus EB injections associated with reinsertion and removal of the P4 insert, resulted in improved d-28 insemination and pregnancy rates and earlier conception compared with no treatment. In contrast, pregnancy rate by d 56 and by the end of the breeding season was not improved by treatment.

Because no interactions were detected between treatment and herd, age groups, postpartum interval, or BCS, it can be concluded that this treatment protocol is effective across a range of ages, breeds, and BCS.

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